IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Sontheimer et al.	§	**ART UNIT: 1642**
	§	
FILED: November 28, 1997	§	**EXAMINER:**
	§	**Sun-Hoffman, L.**
SERIAL NO.: 08/980,395	§	
	§	**DOCKET:**
FOR: Novel Methods of Diagnosing	§	**D5858D1**
and Treating Glioma	§	

The Assistant Commissioner of Patents and Trademarks
BOX AF
Washington, DC 20231

ATTENTION: Board of Patent Appeals and Interferences

APPELLANT'S BRIEF

This Brief is in furtherance of the Notice of Appeal filed in this case on September 23, 1999. The fees required under 37 C.F.R. §1.17(f) and any other required fees are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

In accordance with 37 C.F.R. §1.192(a), this Brief is submitted in triplicate.

INDEX OF SUBJECT MATTER

I. REAL PARTY IN INTEREST

The real party in interest is the University of Alabama Research Foundation, the Assignee, as evidenced by an Assignment recorded in the Patent & Trademark Office at Reel 7805, frame 0893, on February 2, 1996.

II. STATUS OF THE CLAIMS

Originally, claims 1-4 were filed with this Divisional Application. Claims 1 and 4 have been amended. Claims 2 and 3 have been cancelled. The pending claims 1 and 4 are being appealed, of which claim 1 is an independent claim.

III. STATUS OF AMENDMENTS

Claims 1 and 4 were amended in responses filed September 22, 1998 and March 18, 1999 and the amendments were entered. In a Response After Final Office Action filed July 12, 1999,

claim 1 was amended; the Advisory Action mailed September 1, 1999 did not indicate whether the amendment was entered. All pending claims are shown in Appendix A.

IV. STATEMENT OF RELATED APPEALS AND INTERFERENCES

To Appellant's knowledge, there are no pending related appeals or interferences which will directly affect or be directly affected by the present appeal.

V. SUMMARY OF THE INVENTION

Glioma cells, e.g. primary brain tumors derived from glial cells, express a unique membrane protein which constitutes a Cl^- ion channel termed herein glioma chloride channel (Page 13, lines 10-12). This channel can be blocked physiologically by chlorotoxin (Page 48, lines 22-23), a 36 amino acid protein naturally derived from *leiurus quinquestriatus* (Page 49, lines 11-12) scorpion venom known to block epithelial chloride channels (Page 48, lines 23-24).

In the brain, the glioma chloride channel is specific to gliomas and meningiomas and is not present in other cells (Page 13, lines 12-14). Binding is preserved in both synthetic and recombinant forms of chlorotoxin, and also if the molecule is altered in ways to carry fluorescent or cytotoxic moieties (Page 13, lines 20-22). The high affinity for chlorotoxin allows the development of glioma-specific agents including marker compounds for rapid diagnosis and immunotoxins for therapeutic treatment (Page 44, lines 6-8). The present invention provides a pharmaceutical composition, comprising a ligand which binds specifically to glial-derived or meningioma-derived tumor cells (Page 15, lines 3-4) consisting of chlorotoxin ligand and a pharmaceutically acceptable carrier (Page 60, claim 1). In some cases, the chlorotoxin ligand is radiolabeled (Page 60, claim 4).

VI. ISSUES

A. 35 U.S.C. §103

(1) Whether claims 1 and 4 are obvious over **DeBin** *et al.* (Am. J. Physiol. 264/2, 33-2 (C361-C369), 1993) in view of **Weiss** *et al.* (U.S. Patent No: 5,750,376, May 12, 1998 under 35 USC §103(a)).

VII. GROUPING OF CLAIMS

The rejected claims stand or fall together.

VIII. ARGUMENTS

35 U.S.C. §103

Claims 1 and 4 stand rejected under 35 USC §103(a) as being obvious over the combination of **DeBin** *et al.* (Am. J. Physiol. 264/2, 33-2 (C361-C369), 1993) and **Weiss** *et al.* (U.S. Patent No: 5,750,376, May 12, 1998). This rejection is respectfully traversed.

DeBin *et al.* describes the purification and characterization of chlorotoxin from the venom of the *Leiurus quinquestrius* scorpion. It had been demonstrated previously that this venom blocks small conductance Cl^- channels from rat epithelial cells and embryonic brain when applied to the cytoplasmic surface of the channels. To characterize the component responsible for this activity, chlorotoxin was purified and found to be a small peptide of

4070 Da. The purified chlorotoxin was found to block reconstituted rat colonic enterocyte Cl^- channels when applied to the intracellular face of the channels. Extensive kinetic studies of this blocking were undertaken.

The specificity of chlorotoxin for glioma and meningioma cells is not taught by **DeBin** *et al.* In fact, **DeBin** does not describe chlorotoxin specificity for any particular cell type. In addition, **DeBin** *et al.* applied the toxin to the cytoplasmic side of reconstituted rat Cl^- channels. The toxin was never administered to intact mammalian cells. Finally, **DeBin** *et al.* made no proposal to incorporate chlorotoxin into a pharmaceutical composition for any purpose.

Weiss *et al.* teaches a method of producing genetically modified, multipotent neural stem cells, in the course of which **Weiss** labels antibodies for Western blotting, radioimmune assays, and an immunochemistry assay. However, **Weiss** makes no mention of chlorotoxin.

No combination of **DeBin** nor **Weiss** would lead one skilled in the art to incorporate chlorotoxin in a pharmaceutical composition for the specific detection or destruction of glial-derived or meningioma-derived tumor cells. Neither reference teaches that chlorotoxin can distinguish glioma cell and meningioma cells from brain cells. The Examiner has acknowledged that the ability of chlorotoxin to bind glial-derived or meningioma-derived tumor cells was a previously unknown quantity of the protein (Page 2, Second paragraph of the Final Office Action of June 24, 1999). Based on the information available in **DeBin** and **Weiss,** a person having ordinary skill in this art would not have been motivated to determine that chlorotoxin is specific for these tumors nor would a person having ordinary skill in this art have been motivated to prepare a pharmaceutical composition comprising a chlorotoxin ligand and a pharmaceutically acceptable carrier.

The Examiner maintains that "one skilled in the art would have been motivated to make a pharmaceutical composition of chlorotoxin with a carrier, because it is well known for the storage purpose". There are much simpler methods for the stable storage of proteins than incorporation into pharmaceutical compositions. One

skilled in the art would be more inclined to freeze or lyophilyze the protein among other methods.

A pharmaceutical composition is more than just a method of storing a protein. Pharmaceutical compositions are also designed to facilitate drug delivery and dosage among other things. For this reason, Appellants maintain that one skilled in the art would not have been motivated to incorporate chlorotoxin into a pharmaceutical composition unless one intended to use it as such. However, no combination of **DeBin** and **Weiss** suggests any pharmaceutical application for chlorotoxin. Furthermore, while it may have been obvious to label chlorotoxin for using the methods of **Weiss** *et al.* (who uses standard labeling protocols known to those skilled in the art), it would not have been obvious to include the labeled chlorotoxin in a pharmaceutical composition for the detection and treatment glial-derived or meningioma-derived tumor cells since this activity is not suggested by any combination of **DeBin** and **Weiss**.

Obviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent

some teaching, suggestion, or incentive supporting the combination. No such teaching, suggestion, incentive, or even the slightest suggestion to use chlorotoxin in a pharmaceutical composition to target glial-derived or meningioma-derived tumor cells is made by **DeBin** *et al.*, **Weiss** *et al.*, or any combination thereof. Therefore, the Appellants respectfully request that the decision of the Examiner be reversed, and that claims 1 and 4 be allowed.

Respectfully submitted,

Date: Nov 12, 1999

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A

CLAIMS ON APPEAL

1. A pharmaceutical composition, which binds specifically to glial-derived or meningioma-derived tumor cells comprising a chlorotoxin ligand and a pharmaceutically acceptable carrier.

4. The composition of claim 1, wherein said chlorotoxin ligand is radiolabeled.

US005750376A

United States Patent [19]

Weiss et al.

[11] **Patent Number: 5,750,376**

[45] **Date of Patent: May 12, 1998**

[54] IN VITRO GROWTH AND PROLIFERATION OF GENETICALLY MODIFIED MULTIPOTENT NEURAL STEM CELLS AND THEIR PROGENY

[75] Inventors: **Samuel Weiss**; **Brent Reynolds**, both of Alberta, Canada; **Joseph P. Hammang**; **E. Edward Baetge**, both of Barrington, R.I.

[73] Assignee: **NeuroSpheres Holdings Ltd.**, Calgary, Canada

[21] Appl. No.: **483,122**

[22] Filed: **Jun. 7, 1995**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 270,412, Jul. 5, 1994, abandoned, Ser. No. 385,404, Feb. 7, 1995, abandoned, Ser. No. 359,945, Dec. 20, 1994, abandoned, Ser. No. 376,062, Jan. 20, 1995, abandoned, Ser. No. 149,508, Nov. 9, 1993, abandoned, Ser. No. 311,099, Sep. 23, 1994, abandoned, and Ser. No. 338,730, Nov. 14, 1994, abandoned, which is a continuation-in-part of Ser. No. 726,812, Jul. 8, 1991, abandoned, said Ser. No. 385,404, Feb. 7, 1995, abandoned, is a continuation of Ser. No. 961,813, Oct. 16, 1992, abandoned, which is a continuation-in-part of Ser. No. 726,812, Jul. 8, 1991, abandoned, said Ser. No. 359,345, Dec. 20, 1994, abandoned, is a continuation of Ser. No. 221,655, Apr. 1, 1994, abandoned, which is a continuation of Ser. No. 967,622, Oct. 28, 1992, abandoned, which is a continuation-in-part of Ser. No. 726,812, Jul. 8, 1991, abandoned, said Ser. No. 376,062, Jan. 20, 1995, abandoned, is a continuation of Ser. No. 10,829, Jan. 29, 1993, abandoned, which is a continuation-in-part of Ser. No. 726,812, Jul. 8, 1991, abandoned, said Ser. No. 270,412, Jul. 5, 1994, abandoned, Ser. No. 149,508, Nov. 9, 1993, abandoned, and Ser. No. 311,099, Sep. 23, 1994, abandoned, each is a continuation-in-part of Ser. No. 726,812, Jul. 8, 1991, abandoned.

[51] **Int. Cl.**[6] **C12N 5/00**; C12N 5/08; C12N 5/10; C12P 1/00

[52] **U.S. Cl.** **435/69.52**; 435/69.1; 435/172.3; 435/325; 435/368; 435/377; 435/384; 435/392; 435/395

[58] **Field of Search** 435/240.2, 172.3, 435/69.1, 69.52, 325, 368, 377, 384, 392, 395

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(List continued on next page.)

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[57] **ABSTRACT**

A method for producing genetically modified neural cells comprises culturing cells derived from embryonic, juvenile, or adult mammalian neural tissue with one or more growth factors that induce multipotent neural stem cells to proliferate and produce multipotent neural stem cell progeny which include more daughter multipotent neural stem cells and undifferentiated progeny that are capable of differentiating into neurons, astrocytes, and oligodendrocytes. The proliferating neural cells can be transfected with exogenous DNA to produce genetically modified neural stem cell progeny. The genetic modification can be for the production of biologically useful proteins such as growth factor products, growth factor receptors, neurotransmitters, neurotransmitter receptors, neuropeptides and neurotransmitter synthesizing genes. The multipotent neural stem cell progeny can be continuously passaged and proliferation reinitiated in the presence of growth factors to result in an unlimited supply of neural cells for transplantation and other purposes. Culture conditions can be provided that induce the genetically modified multipotent neural stem cell progeny to differentiate into neurons, astrocytes, and oligodendrocytes in vitro.

40 Claims, 3 Drawing Sheets

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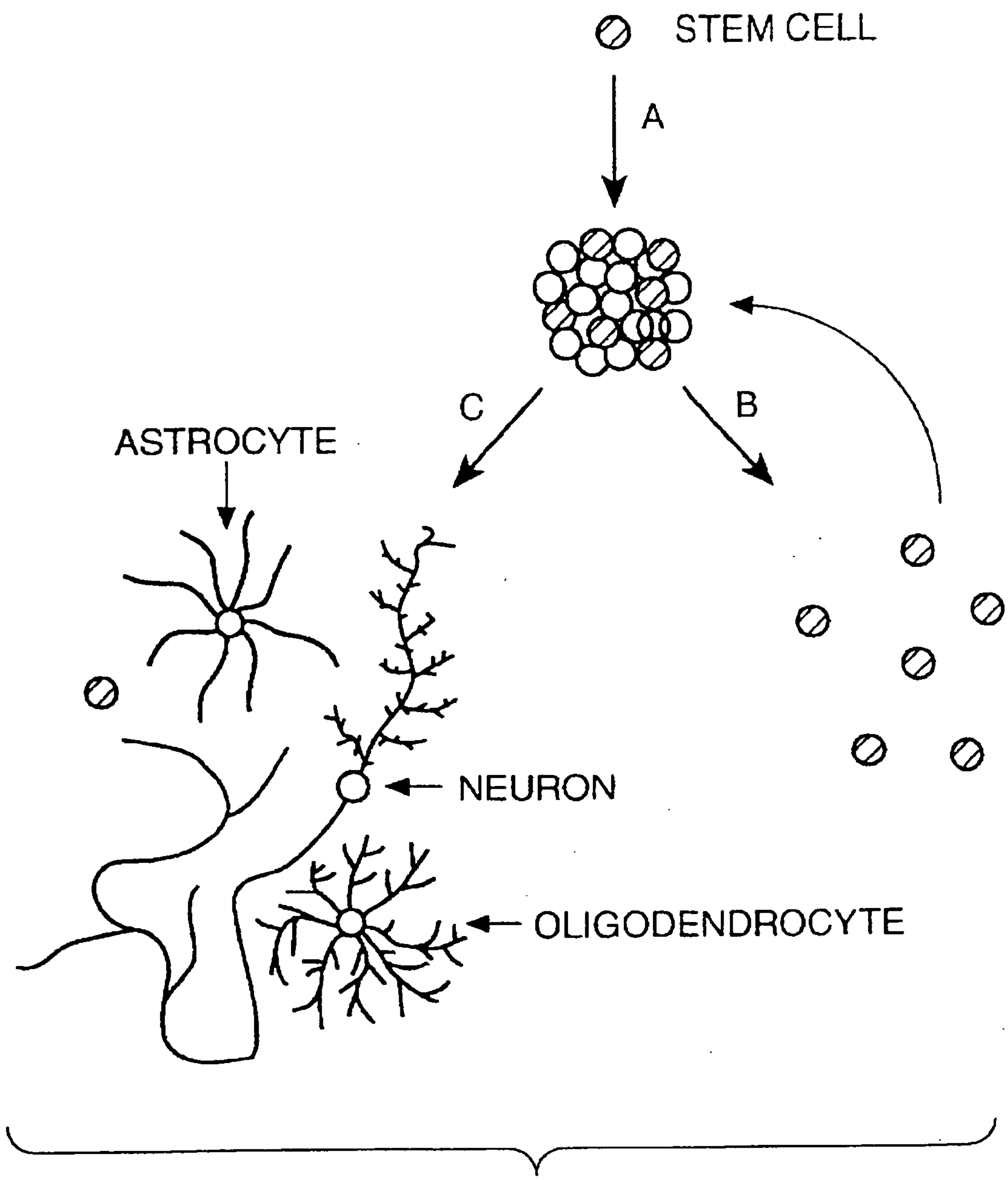

FIG._1

FIG._2A FIG._2B

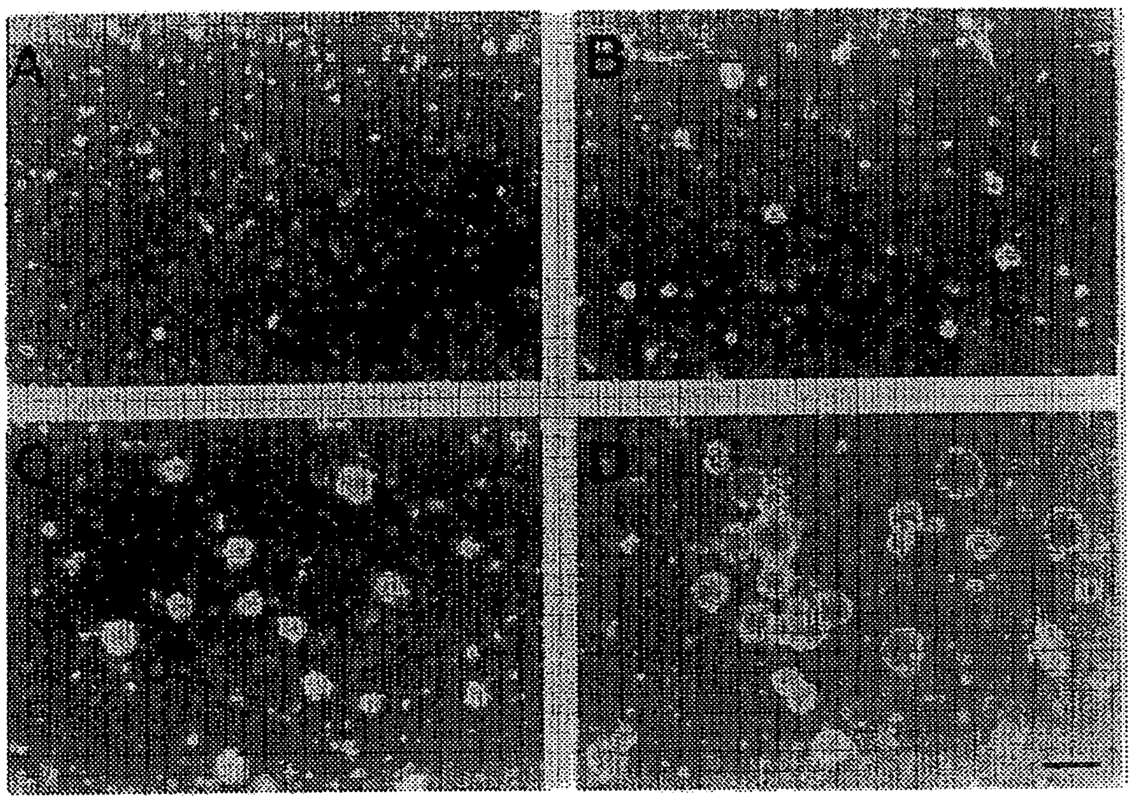

FIG._2C FIG._2D

FIG._3A FIG._3B

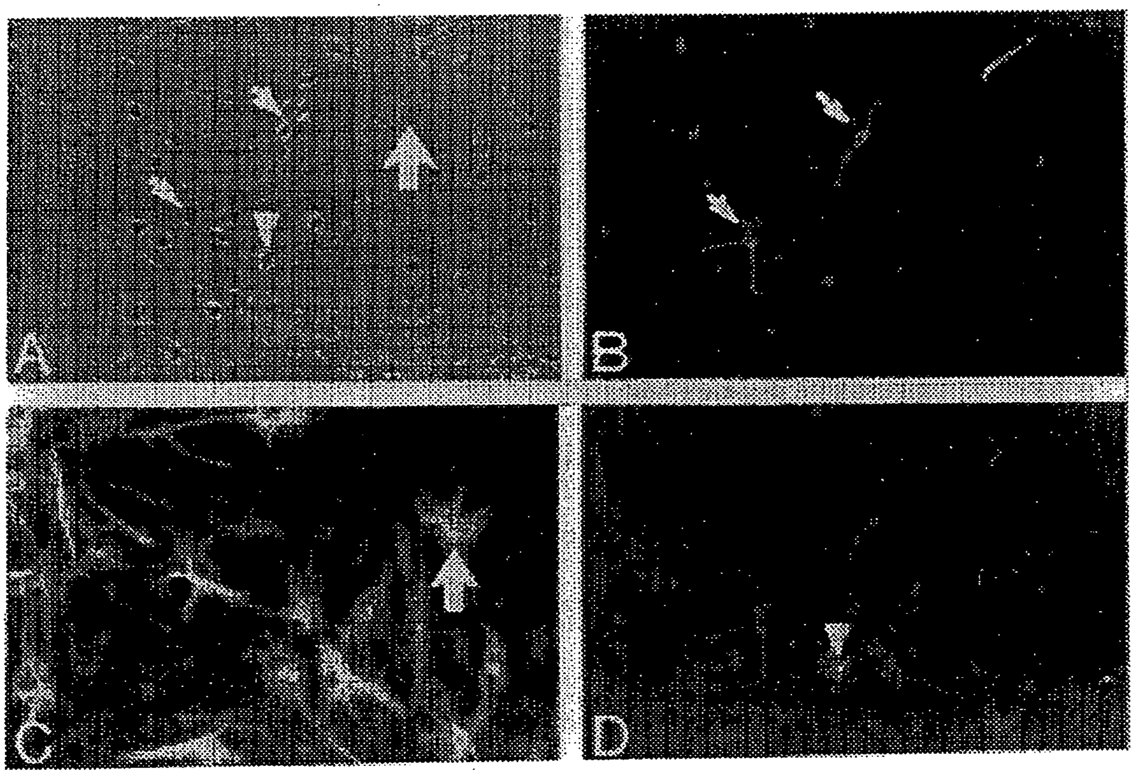

FIG._3C FIG._3D

IN VITRO GROWTH AND PROLIFERATION OF GENETICALLY MODIFIED MULTIPOTENT NEURAL STEM CELLS AND THEIR PROGENY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Ser. No. 08/270,412, filed Jul. 5, 1994, now abandoned, which is a continuation of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; a continuation-in-part of U.S. Ser. No. 08/385,404, filed Feb. 7, 1995, now abandoned, which is a continuation of U.S. Ser. No. 07/961,813, filed Oct. 16, 1992, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; a continuation-in-part of U.S. Ser. No. 08/359,945, filed Dec. 20, 1994, now abandoned, which is a continuation of U.S. Ser. No. 08/221,655, filed Apr. 1, 1994, now abandoned, which is a continuation of U.S. Ser. No. 07/967,622, filed Oct. 28, 1992, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; a continuation-in-part of U.S. Ser. No. 08/376,062, filed Jan. 20, 1995, now abandoned, which is a continuation of U.S. Ser. No. 08/010,829, filed Jan. 29, 1993, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; a continuation-in-part of U.S. Ser. No. 08/149,508, filed Nov. 9, 1993, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; a continuation-in-part of U.S. Ser. No. 08/311,099, filed Sep. 23, 1994, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned; and a continuation-in-part of U.S. Ser. No. 08/338,730, filed Nov. 14, 1994, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/726,812, filed Jul. 8, 1991, now abandoned.

FIELD OF THE INVENTION

This invention relates to a method for the in vitro culture and proliferation of multipotent neural stem cells, and to the use of these cells and their progeny as tissue grafts. In one aspect, this invention relates to a method for the isolation and in vitro perpetuation of large numbers of non-tumorigenic neural stem cell progeny which can be induced to differentiate and which can be used for neurotransplantation in the undifferentiated or differentiated state, into an animal to alleviate the symptoms of neurologic disease, neurodegeneration and central nervous system (CNS) trauma. In another aspect, this invention relates to a method of generating neural cells for the purposes of drug screening of putative therapeutic agents targeted at the nervous system. In another aspect, this invention also relates to a method of generating cells for autologous transplantation. In another aspect, the invention relates to a method for the in vivo proliferation and differentiation of the neural stem cell progeny in the host.

BACKGROUND OF THE INVENTION

The development of the mammalian central nervous system (CNS) begins in the early stage of fetal development and continues until the post-natal period. The mature mammalian CNS is composed of neuronal cells (neurons), and glial cells (astrocytes and oligodendrocytes).

The first step in neural development is cell birth, which is the precise temporal and spatial sequence in which stem cells and stem cell progeny (i.e daughter stem cells and progenitor cells) proliferate. Proliferating cells will give rise to neuroblasts, glioblasts and new stem cells.

The second step is a period of cell type differentiation and migration when undifferentiated progenitor cells differentiate into neuroblasts and glioblasts which give rise to neurons and glial cells which migrate to their final positions. Cells which are derived from the neural tube give rise to neurons and glia of the CNS, while cells derived from the neural crest give rise to the cells of the peripheral nervous system (PNS). Certain factors present during development, such as nerve growth factor (NGF), promote the growth of neural cells. NGF is secreted by cells of the neural crest and stimulates the sprouting and growth of the neuronal axons.

The third step in development occurs when cells acquire specific phenotypic qualities, such as the expression of particular neurotransmitters. At this time, neurons also extend processes which synapse on their targets. Neurons are generated primarily during the fetal period, while oligodendrocytes and astrocytes are generated during the early post-natal period. By the late post-natal period, the CNS has its full complement of nerve cells.

The final step of CNS development is selective cell death, wherein the degeneration and death of specific cells, fibers and synaptic connections "fine-tune" the complex circuitry of the nervous system. This "fine-tuning" continues throughout the life of the host. Later in life, selective degeneration due to aging, infection and other unknown etiologies can lead to neurodegenerative diseases.

Unlike many other cells found in different tissues, the differentiated cells of the adult mammalian CNS have little or no ability to enter the mitotic cycle and generate new nerve cells. While it is believed that there is a limited and slow turnover of astrocytes (Korr et al., J. Comp. Neurol., 150:169, 1971) and that progenitors for oligodendrocytes (Wolsqijk and Noble, Development, 105:386, 1989) are present, the generation of new neurons does not normally occur.

Neurogenesis, the generation of new neurons, is complete early in the postnatal period. However, the synaptic connections involved in neural circuits are continuously altered throughout the life of the individual, due to synaptic plasticity and cell death. A few mammalian species (e.g. rats) exhibit the limited ability to generate new neurons in restricted adult brain regions such as the dentate gyrus and olfactory bulb (Kaplan, J. Comp. Neurol., 195:323, 1981; Bayer, N.Y. Acad. Sci., 457:163, 1985). However, this does not apply to all mammals; and the generation of new CNS cells in adult primates does not occur (Rakic, Science, 227:1054, 1985). This inability to produce new nerve cells in most mammals (and especially primates) may be advantageous for long-term memory retention; however, it is a distinct disadvantage when the need to replace lost neuronal cells arises due to injury or disease.

The low turnover of cells in the mammalian CNS together with the inability of the adult mammalian CNS to generate new neuronal cells in response to the loss of cells following injury or disease has led to the assumption that the adult mammalian CNS does not contain multipotent neural stem cells.

The critical identifying feature of a stem cell is its ability to exhibit self-renewal or to generate more of itself. The simplest definition of a stem cell would be a cell with the capacity for self-maintenance. A more stringent (but still simplistic) definition of a stem cell is provided by Potten and Loeffler (Development, 110:1001, 1990) who have defined stem cells as "undifferentiated cells capable of a)

proliferation, b) self-maintenance, c) the production of a large number of differentiated functional progeny, d) regenerating the tissue after injury, and e) a flexibility in the use of these options."

The role of stem cells is to replace cells that are lost by natural cell death, injury or disease. The presence of stem cells in a particular type of tissue usually correlates with tissues that have a high turnover of cells. However, this correlation may not always hold as stem cells are thought to be present in tissues (e.g., liver [Travis, Science, 259:1829, 1993]) that do not have a high turnover of cells.

CNS disorders encompass numerous afflictions such as neurodegenerative diseases (e.g. Alzheimer's and Parkinson's), acute brain injury (e.g. stroke, head injury, cerebral palsy) and a large number of CNS dysfunctions (e.g. depression, epilepsy, and schizophrenia). In recent years neurodegenerative disease has become an important concern due to the expanding elderly population which is at greatest risk for these disorders. These diseases, which include Alzheimer's Disease, Multiple Sclerosis (MS), Huntington's Disease, Amyotrophic Lateral Sclerosis, and Parkinson's Disease, have been linked to the degeneration of neural cells in particular locations of the CNS, leading to the inability of these cells or the brain region to carry out their intended function.

In addition to neurodegenerative diseases, acute brain injuries often result in the loss of neural cells, the inappropriate functioning of the affected brain region, and subsequent behavior abnormalities. Probably the largest area of CNS dysfunction (with respect to the number of affected people) is not characterized by a loss of neural cells but rather by an abnormal functioning of existing neural cells. This may be due to inappropriate firing of neurons, or the abnormal synthesis, release, and processing of neurotransmitters. These dysfunctions may be the result of well studied and characterized disorders such as depression and epilepsy, or less understood disorders such as neurosis and psychosis.

Degeneration in a brain region known as the basal ganglia can lead to diseases with various cognitive and motor symptoms, depending on the exact location. The basal ganglia consists of many separate regions, including the striatum (which consists of the caudate and putamen), the globus pallidus, the substantia nigra, substantia innominate, ventral pallidum, nucleus basalis of Meynert, ventral tegmental area and the subthalamic nucleus.

In the case of Alzheimer's Disease, there is a profound cellular degeneration of the forebrain and cerebral cortex. In addition, upon closer inspection, a localized degeneration in an area of the basal ganglia, the nucleus basalis of Meynert, appears to be selectively degenerated. This nucleus normally sends cholinergic projections to the cerebral cortex which are thought to participate in cognitive functions including memory.

Many motor deficits are a result of degeneration in the basal ganglia. Huntington's Chorea is associated with the degeneration of neurons in the striatum, which leads to involuntary jerking movements in the host. Degeneration of a small region called the subthalamic nucleus is associated with violent flinging movements of the extremities in a condition called ballismus, while degeneration in the putamen and globus pallidus is associated with a condition of slow writhing movements or athetosis. In the case of Parkinson's Disease, degeneration is seen in another area of the basal ganglia, the substantia nigra par compacta. This area normally sends dopaminergic connections to the dorsal striatum which are important in regulating movement. Therapy for Parkinson's Disease has centered upon restoring dopaminergic activity to this circuit.

Other forms of neurological impairment can occur as a result of neural degeneration, such as amyotrophic lateral sclerosis and cerebral palsy, or as a result of CNS trauma, such as stroke and epilepsy.

Demyelination of central and peripheral neurons occurs in a number of pathologies and leads to improper signal conduction within the nervous systems. Myelin is a cellular sheath, formed by glial cells, that surrounds axons and axonal processes that enhances various electrochemical properties and provides trophic support to the neuron. Myelin is formed by Schwann cells in the PNS and by oligodendrocytes in the CNS. Among the various demyelinating diseases MS is the most notable.

To date, treatment for CNS disorders has been primarily via the administration of pharmaceutical compounds. Unfortunately, this type of treatment has been fraught with many complications including the limited ability to transport drugs across the blood-brain barrier and the drug-tolerance which is acquired by patients to whom these drugs are administered long-term. For instance, partial restoration of dopaminergic activity in Parkinson's patients has been achieved with levodopa, which is a dopamine precursor able to cross the blood-brain barrier. However, patients become tolerant to the effects of levodopa, and therefore, steadily increasing dosages are needed to maintain its effects. In addition, there are a number of side effects associated with levodopa such as increased and uncontrollable movement.

Recently, the concept of neurological tissue grafting has been applied to the treatment of neurological diseases such as Parkinson's Disease. Neural grafts may avert the need not only for constant drug administration, but also for complicated drug delivery systems which arise due to the blood-brain barrier. However, there are limitations to this technique as well. First, cells used for transplantation which carry cell surface molecules of a differentiated cell from another host can induce an immune reaction in the host. In addition, the cells must be at a stage of development where they are able to form normal neural connections with neighboring cells. For these reasons, initial studies on neurotransplantation centered on the use of fetal cells. Perlow, et al. describe the transplantation of fetal dopaminergic neurons into adult rats with chemically induced nigrostriatal lesions in "Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system," Science 204:643–647 (1979). These grafts showed good survival, axonal outgrowth and significantly reduced the motor abnormalities in the host animals.

In both human demyelinating diseases and rodent models there is substantial evidence that demyelinated neurons are capable of remyelination in vivo. In MS, for example, it appears that there are often cycles of de- and remyelination. Similar observations in rodent demyelinating paradigms lead to the prediction that exogenously applied cells would be capable of remyelinating demyelinated axons. This approach has proven successful in a number of experimental conditions [Freidman et al., *Brain Research,* 378:142–146 (1986); Raine, et al., *Laboratory Investigation* 59:467–476 (1988); Duncan et al., *J. of Neurocytology,* 17:351–360 (1988)]. The sources of cells for some of these experiments included dissociated glial cell suspensions prepared from spinal cords (Duncan et al., supra), Schwann cell cultures prepared from sciatic nerve [Bunge et al., 1992, WO 92/03536; Blakemore and Crang, *J. Neurol. Sci.,* 70:207–223 (1985)]; cultures from dissociated brain tissue

[Blakemore and Crang, *Dev. Neurosci.* 10:1–11 (1988)], oligodendrocyte precursor cells [Gumpel et al., *Dev. Neurosci.* 11:132–139 (1989)], O-2A cells [Wolswijk et al., *Development* 109:691–608 (1990); Raff et al., *Nature* 3030:390–396 (1983); Hardy et al., *Development* 111:1061–1080 (1991)], and immortalized O-2A cell lines, [Almazan and McKay *Brain Res.* 579:234–245 (1992)].

O-2A cells are glial progenitor cells which give rise in vitro only to oligodendrocytes and type II astrocytes. Cells which appear by immunostaining in vivo to have the O-2A phenotype have been shown to successfully remyelinate demyelinated neurons in vivo, [Godfraind et al., *J. Cell Biol.* 109:2405–2416 (1989)]. Injection of a large number of O-2A cells is required to adequately remyelinate all targeted neurons in vivo, since it appears that O-2A cells (like other glial cell preparations) do not continue to divide in vivo. Although O-2A progenitor cells can be grown in culture, currently the only available isolation technique employs optic nerve as starting material. This is a low yield source, which requires a number of purification steps. There is an additional drawback that O-2A cells isolated by the available procedures are capable of only a limited number of divisions [Raff *Science* 243:1450–1455 (1989)].

Although adult CNS neurons are not good candidates for neurotransplantation, neurons from the adult PNS have been shown to survive transplantation, and to exert neurotrophic and gliotrophic effects on developing host neural tissue. One source of non-CNS neural tissue for transplantation is the adrenal medulla.

Adrenal chromaffin cells originate from the neural crest like PNS neurons, and receive synapses and produce carrier and enzyme proteins similar to PNS neurons.

Although these cells function in an endocrine manner in the intact adrenal medulla, in culture these cells lose their glandular phenotype and develop certain neural features in culture in the presence of certain growth factors and hormones [Notter, et al., "Neuronal properties of monkey adrenal medulla in vitro, *Cell Tissue Research* 244:69–76 (1986)]. When grafted into mammalian CNS, these cells survive and synthesize significant quantities of dopamine which can interact with dopamine receptors in neighboring areas of the CNS.

In U.S. Pat. No. 4,980,174, transplantation of monoamine-containing cells isolated from adult rat pineal gland and adrenal medulla into rat frontal cortex led to the alleviation of learned helplessness, a form of depression in the host. In U.S. Pat. No. 4,753,635, chromaffin cells and adrenal medullary tissue derived from steers were implanted into the brain stem or spinal cord of rats and produced analgesia when the implanted tissue or cell was induced to release nociceptor interacting substances (i.e. catecholamines such as dopamine). Adrenal medullary cells have been autologously grafted into humans, and have survived, leading to mild to moderate improvement in symptoms (Watts, et al., "Adrenal-caudate transplantation in patients with Parkinson's Disease (PD):1-year follow-up," *Neurology* 39 Suppl 1: 127 [1989], Hurtig, et al., "Postmortem analysis of adrenal-medulla-to-caudate autograft in a patient with Parkinson's Disease," *Annals of Neurology* 25: 607–614 [1989]). However, adrenal cells do not obtain a normal neural phenotype, and are therefore probably of limited use for transplants where synaptic connections must be formed.

Another source of tissue for neurotransplantation is from cell lines. Cell lines are immortalized cells which are derived either by transformation of normal cells with an oncogene (Cepko, "Immortalization of neural cells via retrovirus-mediated oncogene transduction," *Ann. Rev. Neurosci.* 12:47–65 [1989]) or by the culturing of cells with altered growth characteristics in vitro (Ronnett, et al., "Human cortical neuronal cell line: Establishment from a patient with unilateral megalencephaly," *Science* 248:603–605 [1990]). Such cells can be grown in culture in large quantities to be used for multiple transplantations. Some cell lines have been shown to differentiate upon chemical treatment to express a variety of neuronal properties such as neurite formation, excitable membranes and synthesis of neurotransmitters and their receptors. Furthermore, upon differentiation, these cells appear to be amitotic, and therefore noncancerous. However, the potential for these cells to induce adverse immune responses, the use of retroviruses to immortalize cells, the potential for the reversion of these cells to an amitotic state, and the lack of response of these cells to normal growth-inhibiting signals make cell lines less than optimal for widespread use.

Another approach to neurotransplantation involves the use of genetically engineered cell types or gene therapy. Using this method, a foreign gene or transgene can be introduced into a cell which is deficient in a particular enzymatic activity, thereby allowing the cell to express the gene. Cells which now contain the transferred gene can be transplanted to the site of neurodegeneration, and provide products such as neurotransmitters and growth factors (Rosenberg, et al., "Grafting genetically modified cells to the damaged brain: Restorative effects of NGF Expression," *Science* 242:1575–1578, [1988]) which may function to alleviate some of the symptoms of degeneration. However, there still exists a risk of inducing an immune reaction using currently available cell lines. In addition, these cells may also not achieve normal neuronal connections with the host tissue.

Genetically modified cells have been used in neurological tissue grafting in order to replace lost cells which normally produce a neurotransmitter. For example, fibroblasts have been genetically modified with a retroviral vector containing a cDNA for tyrosine hydroxylase, which allows them to produce dopamine, and implanted into animal models of Parkinson's Disease (Gage et al., U.S. Pat. No. 5,082,670).

While the use of genetically modified fibroblasts to treat CNS disorders has shown promise in improving some behavioral deficits in animal models of Parkinson's Disease, and represents a novel approach to supplying a needed transmitter to the CNS, it suffers from several significant drawbacks as a treatment for Parkinson's Disease and in general as a therapeutic approach for treating neurodegenerative diseases and brain injury. First, the CNS is primarily composed of three cell types—neurons, astrocytes and oligodendrocytes. The implantation of a foreign cell such as a fibroblast into the CNS and its direct and indirect effects on the functioning of the host cells has yet to be studied. However, it is likely that the expression of membrane bound factors and the release of soluble molecules such as growth factors and proteases will alter the normal behavior of the surrounding tissue. This may result in the disruption of neuronal firing patterns either by a direct action on neurons or by an alteration in the normal functioning of glial cells.

Another concern that arises when fibroblasts are implanted into the CNS is the possibility that the implanted cells may lead to tumor formation because the intrinsic inhibition of fibroblast division is poorly controlled. Instead, extrinsic signals play a major role in controlling the number of divisions the cell will undergo. The effect of the CNS environment on the division of implanted fibroblasts and the

high probability of a fibroblastic tumor formation has not been studied in the long-term.

A third concern in transplanting fibroblasts into the CNS is that fibroblasts are unable to integrate with the CNS cells as astrocytes, oligodendrocytes, or neurons do. Fibroblasts are intrinsically limited in their ability to extend neuronal-like processes and form synapses with host tissue. Hence, although the genetic modification and implantation of fibroblasts into the CNS represents an improvement over the current technology for the delivery of certain molecules to the CNS, the inability of fibroblasts to integrate and function as CNS tissue, their potential negative effects on CNS cells, and their limited intrinsic control of proliferation limits their practical usage for implantation for the treatment of acute or chronic CNS injury or disease.

A preferred tissue for genetic modification and implantation would be CNS cells—neurons, astrocytes, or oligodendrocytes. One source of CNS cells is from human fetal tissue. Several studies have shown improvements in patients with Parkinson's Disease after receiving implants of fetal CNS tissue. Implants of embryonic mesencephalic tissue containing dopamine cells into the caudate and putamen of human patients was shown by Freed et al. (*N Engl J Med* 327:1549–1555 (1992)) to offer long-term clinical benefit to some patients with advanced Parkinson's Disease. Similar success was shown by Spencer et al. (*N Engl J Med* 327:1541–1548 (1992)). Widner et al. (*N Engl J Med* 327:1556–1563 (1992)) have shown long-term functional improvements in patients with MPTP-induced Parkinsonism that received bilateral implantation of fetal mesencephalic tissue.

While the studies noted above are encouraging, the use of large quantities of aborted fetal tissue for the treatment of disease raises ethical considerations and political obstacles. There are other considerations as well. Fetal CNS tissue is composed of more than one cell type, and thus is not a well-defined source of tissue. In addition, there are serious doubts as to whether an adequate and constant supply of fetal tissue would be available for transplantation. For example, in the treatment of MPTP-induced Parkinsonism (Widner supra) tissue from 6 to 8 fetuses were used for implantation into the brain of a single patient. There is also the added problem of the potential for contamination during fetal tissue preparation. Moreover, the tissue may already be infected with a bacteria or virus, thus requiring expensive diagnostic testing for each fetus used. However, even diagnostic testing might not uncover all infected tissue. For example, the diagnosis of HIV-free tissue is not guaranteed because antibodies to the virus are generally not present until several weeks after infection.

While currently available transplantation approaches represent a significant improvement over other available treatments for neurological disorders, they suffer from significant drawbacks. The inability in the prior art of the transplant to fully integrate into the host tissue, and the lack of availability of cells in unlimited amounts from a reliable source for grafting are, perhaps, the greatest limitations of neurotransplantation.

It would be more preferable to have a well-defined, reproducible source of neural tissue for transplantation that is available in unlimited amounts. Since adult neural tissue undergoes minimal division, it does not readily meet these criteria. While astrocytes retain the ability to divide and are probably amenable to infection with foreign genes, their ability to form synapses with neuronal cells is limited and consequently so is their extrinsic regulation of the expression and release of the foreign gene product.

Oligodendrocytes suffer from some of the same problems. In addition, mature oligodendrocytes do not divide, limiting the infection of oligodendrocytes to their progenitor cells (e.g. O2A cells). However, due to the limited proliferative ability of oligodendrocyte progenitors, the infection and harvesting of these cells does not represent a practical source.

The infection of neurons with foreign genes and implantation into the CNS would be ideal due to their ability to extend processes, make synapses and be regulated by the environment. However, differentiated neurons do not divide and transfection with foreign genes by chemical and physical means is not efficient, nor are they stable for long periods of time. The infection of primary neuronal precursors with retroviral vectors in vitro is not practical either because neuroblasts are intrinsically controlled to undergo a limited number of divisions making the selection of a large number of neurons, that incorporate and express the foreign gene, nearly impossible. The possibility of immortalizing the neuronal precursors by retroviral transfer of oncogenes and their subsequent infection of a desired gene is not preferred due to the potential for tumor formation by the implanted cells.

In addition to the need for a well-defined, reproducible source of neural cells available in unlimited amounts for transplantation purposes, a similar need exists for drug screening purposes and for the study of CNS function, dysfunction, and development. The mature human nervous system is composed of billions of cells that are generated during development from a small number of precursors located in the neural tube. Due to the complexity of the mammalian CNS, the study of CNS developmental pathways, as well as alterations that occur in adult mammalian CNS due to dysfunction, has been difficult. Such areas would be better studied using relatively simple models of the CNS under defined conditions.

Generally, two approaches have been taken for studying cultured CNS cells: the use of primary neural cultures; and the use of neural cell lines. Primary mammalian neural cultures can be generated from nearly all brain regions providing that the starting material is obtained from fetal or early post-natal animals. In general, three types of cultures can be produced, enriched either in neurons, astrocytes, or oligodendrocytes. Primary CNS cultures have proven valuable for discovering many mechanisms of neural function and are used for studying the effects of exogenous agents on developing and mature cells. While primary CNS cultures have many advantages, they suffer from two primary drawbacks. First, due to the limited proliferative ability of primary neural cells, new cultures must be generated from several different animals. While great care is usually taken to obtain tissue at identical states of development and from identical brain regions, it is virtually impossible to generate primary cultures that are identical. Hence, there exists a significant degree of variability from culture to culture.

A second disadvantage of primary cultures is that the tissue must be obtained from fetuses or early post-natal animals. If primary cultures are to be performed on a regular basis, this requires the availability of a large source of starting material. While this is generally not a problem for generating primary cultures from some species (e.g. rodents), it is for others (e.g. primates). Due to the limited supply and ethical concerns, the culturing of primary cells from primates (both human and non-human) is not practical.

Due to the limited proliferative ability of primary neural cells, the generation of a large number of homogenous cells

for studies of neural function, dysfunction, and drug design/screening has previously not been achieved. Therefore, homogenous populations of cells that can generate a large number of progeny for the in vitro investigation of CNS function has been studied by the use of cell lines. The generation of neural cell lines can be divided into two categories: 1) spontaneously occurring tumors, and 2) custom-designed cell lines.

Of the spontaneously occurring tumors, probably the most studied cell line for neurobiology is the rat pheochromocytoma (PC12) cells that can differentiate into sympathetic-like neurons in response to NGF. These cells have proven to be a useful model for studying mechanisms of neural development and alterations (molecular and cellular) in response to growth factors. Neuroblastoma and glioma cell lines have been used to study neuronal and glial functioning [Liles, et al., *J. Neurosci.* 7, 2556–2563 (1987); Nister et al. *Cancer Res.* 48(14) 3910 (1988)]. Embryonal carcinoma cells are derived from teratoma tumors of fetal germ cells and have the ability to differentiate into a large number of non-neural cell types with some lines (e.g. P19 cells) [Jones-Villeneuve et al. *J. Cell Biol.* 94, 253–262 (1982)] having the ability to differentiate into neural cells [(McBurney et al. *J. Neurosci.* 8(3) 1063–73 (1993)]. A human teratocarcinoma-derived cell line, NTera 2/cl.D1, with a phenotype resembling CNS neuronal precursor cells, can be induced to differentiate in the presence of retinoic acid. However, the differentiated cells are restricted to a neuronal phenotype [Pleasure and Lee *J. Neurosci. Res.* 35: 585–602 (1993)]. While these types of cell lines are able to generate a large number of cells for screening the effects of exogenous agents on cell survival or function, the limited number of these types of lines, the limited number of phenotypes that they are able to generate and the unknown nature of their immortalization (which may effect the function of the cells in an undefined manner) makes these types of cell lines less than ideal for in vitro models of neural function and discovery of novel therapeutics.

An alternative approach to spontaneously occurring cell lines is the intentional immortalization of a primary cell by introducing an oncogene that alters the genetic make-up of the cell thereby inducing the cell to proliferate indefinitely. This approach has been used by many groups to generate a number of interesting neural cell lines [(Bartlett et al. *Proc. Nat. Acad. Sci.* 85(9) 3255–3259 (1988); Frederiksen et al. *Neuron* 1, 439–448 (1988); Trotter et al. *Oncogene* 4: 457–464 (1989); Ryder et al. *J. Neurobiol.* 21: 356–375 (1980); Murphy et al. *J. Neurobiol* 22: 522–535 (1991); Almazan and McKay et al. *Brain Res.* 579: 234–245 (1992)]. While these lines may prove useful for studying the decisions that occur during cell determination and differentiation, and for testing the effects of exogenous agents, they suffer from several drawbacks. First, the addition of an oncogene that alters the proliferative status of a cell may affect other properties of the cell (oncogenes may play other roles in cells besides regulating the cell cycle). This is well illustrated in a study by Almazan and McKay, supra, and their immortalization of an oligodendrocyte precursor from the optic nerve which is unable to differentiate into type II astrocytes (something that normal optic nerve oligodendrocyte precursors can do). The authors suggest the presence of the immortalizing antigen may alter the cells ability to differentiate into astrocytes.

Another drawback to using intentionally immortalized cells results from the fact that the nervous system is composed of billions of cells and possibly thousands of different cell types, each with unique patterns of gene expression and responsiveness to their environment. A custom-designed cell line is the result of the immortalization of a single progenitor cell and its clonal expansion. While a large supply of one neural cell type can be generated, this approach does not take into account cellular interactions between different cell types. In addition, while it is possible to immortalize cells from a given brain region, immortalization of a desired cell is not possible due to the lack of control over which cells will be altered by the oncogene. Hence, while custom designed cell lines offer a few advantages over spontaneously occurring tumors, they suffer from several drawbacks and are less than ideal for understanding CNS function and dysfunction.

Therefore, in view of the aforementioned deficiencies attendant with prior art methods of neural cell culturing, transplantation, and CNS models, a need exists in the art for a reliable source of unlimited numbers of undifferentiated neural cells for neurotransplantation and drug screening which are capable of differentiating into neurons, astrocytes, and oligodendrocytes. Preferably cellular division in such cells from such a source would be epigenetically regulated and a suitable number of cells could be efficiently prepared in sufficient numbers for transplantation. The cells should be suitable in autografts, xenografts, and allografts without a concern for tumor formation. There exists a need for the isolation, perpetuation and transplantation of autologous neural cells from the juvenile or adult brain that are capable of differentiating into neurons and glia.

A need also exists for neural cells, capable of differentiating into neurons, astrocytes and oligodendrocytes that are capable of proliferation in vitro and thus amenable to genetic modification techniques.

Additionally, there exists a need for the repair of damaged neural tissue in a relatively non-invasive fashion, that is by inducing neural cells to proliferate and differentiate into neurons, astrocytes, and oligodendrocytes in vivo, thereby averting the need for transplantation.

Accordingly, a major object of the present invention is to provide a reliable source of an unlimited number of neural cells for neurotransplantation that are capable of differentiating into neurons, astrocytes, and oligodendrocytes.

It is another object of the present invention to provide a method for the in vitro proliferation of neural stem cells from embryonic, juvenile and adult brain tissue, to produce unlimited numbers of precursor cells available for transplantation that are capable of differentiating into neurons, astrocytes, and oligodendrocytes. A further object of the invention is to provide methods for inducing neural cells to proliferate and differentiate in vivo, thereby averting the need for neurotransplantation.

A still further object of the invention is to provide a method of generating large numbers of normal neural cells for the purpose of screening putative therapeutic agents targeted at the nervous system and for models of CNS development, function, and dysfunction.

SUMMARY OF THE INVENTION

This invention provides in one aspect a composition for inducing the proliferation of a multipotent neural stem cell comprising a culture medium supplemented with at least one growth factor, preferably epidermal growth factor or transforming growth factor alpha.

The invention also provides a method for the in vitro proliferation and differentiation of neural stem cells and stem cell progeny comprising the steps of (a) isolating the cell from a mammal, (b) exposing the cell to a culture medium containing a growth factor, (c) inducing the cell to

proliferate, and (d) inducing the cell to differentiate. Proliferation and perpetuation of the neural stem cell progeny can be carried out either in suspension cultures, or by allowing cells to adhere to a fixed substrate. Proliferation and differentiation can be done before or after transplantation, and in various combinations of in vitro or in vivo conditions, including (1) proliferation and differentiation in vitro, then transplantation, (2) proliferation in vitro, transplantation, then further proliferation and differentiation in vivo, and (3) proliferation in vitro, transplantation and differentiation in vivo.

The invention also provides for the proliferation and differentiation of the progenitor cells in vivo, which can be done directly in the host without the need for transplantation.

The invention also provides a method for the in vivo transplantation of neural stem cell progeny, treated as in any of (1) through (3) above, which comprises implanting, into a mammal, these cells which have been treated with at least one growth factor.

Furthermore, the invention provides a method for treating neurodegenerative diseases comprising administering to a mammal neural stem cell progeny which have been treated as in any of (1) through (3), and induced to differentiate into neurons and/or glia.

The invention also provides a method for treating neurodegenerative disease comprising stimulating in vivo mammalian CNS neural stem cells to proliferate and the neural stem cell progeny to differentiate into neurons and/or glia. The invention also provides a method for the transfection of neural stem cells and stem cell progeny with vectors which can express the gene products for growth factors, growth factor receptors, and peptide neurotransmitters, or express enzymes which are involved in the synthesis of neurotransmitters, including those for amino acids, biogenic amines and neuropeptides, and for the transplantation of these transfected cells into regions of neurodegeneration.

In a still further aspect, the invention provides a method for the screening of potential neurologically therapeutic pharmaceuticals using neural stem cell progeny which have been proliferated in vitro.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A–C: Diagram Illustrating the Proliferation of a Multipotent Neural Stem Cell

(A) In the presence of a proliferation-inducing growth factor the stem cell divides and gives rise to a sphere of undifferentiated cells composed of more stem cells and progenitor cells. (B) When the clonally derived sphere of undifferentiated cells is dissociated and plated as single cells, on a non-adhesive substrate and in the presence of a proliferation-inducing growth factor, each stem cell will generate a new sphere. (C) If the spheres are cultured in conditions that allow differentiation, the progenitor cells differentiate into neurons, astrocytes and oligodendrocytes.

FIG. 2A–D: Proliferation Of Epidermal Growth Factor (EGF) Responsive Cells

After 2 days in vitro EGF-responsive cells begin to proliferate (FIG. 2A). After 4 days in vitro small clusters of cells known as neurospheres are apparent (FIG. 2B). The neurospheres of continuously proliferating cells continue to grow in size (FIG. 2C) until they lift off the substrate and float in suspension (FIG. 2D). At this stage, the floating spheres can be easily removed, dissociated into single cells and, in the presence of EGF, proliferation can be re-initiated. (Bar: 50 μm).

FIG. 3A–D: Differentiation Of Cells From Single EGF-Generated Spheres Into Neurons, Astrocytes, And Oligodendrocytes

Triple-label immunocytochemistry with antibodies to microtubule associated protein (MAP-2), glial fibrillary acidic protein (GFAP), and O4 (a cell surface antigen) are used to detect the presence of neurons (FIG. 3B), astrocytes (FIG. 3C) and oligodendrocytes (FIG. 3D), respectively, from an EGF-generated, stem cell-derived neurosphere (FIG. 3A) derived from primary culture. (Bar: 50 μm).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for inducing multipotent neural stem cells from fetal, juvenile, or adult mammalian tissue to proliferate in vitro or in vivo (i.e. in situ), to generate large numbers of neural stem cell progeny capable of differentiating into neurons, astrocytes, and oligodendrocytes. Methods for differentiation of the neural stem cell progeny are also provided. The induction of proliferation and differentiation of neural stem cells can be done either by culturing the cells in suspension or on a substrate onto which they can adhere. Alternatively, proliferation and differentiation of neural stem cells can be induced, under appropriate conditions, in the host in the following combinations: (1) proliferation and differentiation in vitro, then transplantation, (2) proliferation in vitro, transplantation, then further proliferation and differentiation in vivo, (3) proliferation in vitro, transplantation and differentiation in vivo, and (4) proliferation and differentiation in vivo. Proliferation and differentiation in vivo (i.e. in situ) can involve a non-surgical approach that coaxes neural stem cells to proliferate in vivo with pharmaceutical manipulation. Thus, the invention provides a means for generating large numbers of undifferentiated and differentiated neural cells for neurotransplantation into a host in order to treat neurodegenerative disease and neurological trauma, for non-surgical methods of treating neurodegenerative disease and neurological trauma, and for drug-screening applications.

Multipotent Neural Stem Cells

Neurobiologists have used various terms interchangeably to describe the undifferentiated cells of the CNS. Terms such as "stem cell", "precursor cell" and "progenitor cell" are commonly used in the scientific literature. However, there are different types of undifferentiated neural cells, with differing characteristics and fates. U.S. Ser. No. 08/270,412 which is a continuation application of U.S. Ser. No. 07/726,812, termed the cells obtained and proliferated using the methods of Examples 1–4 below "progenitor cells". The terminology used for undifferentiated neural cells has evolved such that these cells are now termed "neural stem cells". U.S. Ser. No. 08/270,412 defines the "progenitor" cell proliferated in vitro to mean "an oligopotent or multipotent stem cell which is able to divide without limit and under specific conditions can produce daughter cells which terminally differentiate into neurons and glia." The capability of a cell to divide without limit and produce daughter cells which terminally differentiate into neurons and glia are stem cell characteristics. Accordingly, as used herein, the cells proliferated using the methods described in Examples 1–4 are termed "neural stem cells". A neural stem cell is an undifferentiated neural cell that can be induced to proliferate using the methods of the present invention. The neural stem cell is capable of self-maintenance, meaning that with each cell division, one daughter cell will also be a stem cell. The

non-stem cell progeny of a neural stem cell are termed progenitor cells. The progenitor cells generated from a single multipotent neural stem cell are capable of differentiating into neurons, astrocytes (type I and type II) and oligodendrocytes. Hence, the neural stem cell is "multipotent" because its progeny have multiple differentiative pathways.

The term "neural progenitor cell", as used herein, refers to an undifferentiated cell derived from a neural stem cell, and is not itself a stem cell. Some progenitor cells can produce progeny that are capable of differentiating into more than one cell type. For example, an O-2A cell is a glial progenitor cell that gives rise to oligodendrocytes and type II astrocytes, and thus could be termed a "bipotential" progenitor cell. A distinguishing feature of a progenitor cell is that, unlike a stem cell, it has limited proliferative ability and thus does not exhibit self-maintenance. It is committed to a particular path of differentiation and will, under appropriate conditions, eventually differentiate into glia or neurons.

The term "precursor cells", as used herein, refers to the progeny of neural stem cells, and thus includes both progenitor cells and daughter neural stem cells.

Neural stem cell progeny can be used for transplantation into a heterologous, autologous, or xenogeneic host. Multipotent neural stem cells can be obtained from embryonic, post-natal, juvenile or adult neural tissue. The neural tissue can be obtained from any animal that has neural tissue such as insects, fish, reptiles, birds, amphibians, mammals and the like. The preferred source neural tissue is from mammals, preferably rodents and primates, and most preferably, mice and humans.

In the case of a heterologous donor animal, the animal may be euthanized, and the neural tissue and specific area of interest removed using a sterile procedure. Areas of particular interest include any area from which neural stem cells can be obtained that will serve to restore function to a degenerated area of the host's nervous system, particularly the host's CNS. Suitable areas include the cerebral cortex, cerebellum, midbrain, brainstem, spinal cord and ventricular tissue, and areas of the PNS including the carotid body and the adrenal medulla. Preferred areas include regions in the basal ganglia, preferably the striatum which consists of the caudate and putamen, or various cell groups such as the globus pallidus, the subthalamic nucleus, the nucleus basalis which is found to be degenerated in Alzheimer's Disease patients, or the substantia nigra pars compacta which is found to be degenerated in Parkinson's Disease patients. Particularly preferred neural tissue is obtained from ventricular tissue that is found lining CNS ventricles and includes the subependyma. The term "ventricle" refers to any cavity or passageway within the CNS through which cerebral spinal fluid flows. Thus, the term not only encompasses the lateral, third, and fourth ventricles, but also encompasses the central canal, cerebral aqueduct, and other CNS cavities.

Human heterologous neural stem cells may be derived from fetal tissue following elective abortion, or from a post-natal, juvenile or adult organ donor. Autologous neural tissue can be obtained by biopsy, or from patients undergoing neurosurgery in which neural tissue is removed, for example, during epilepsy surgery, temporal lobectomies and hippocampalectomies. Neural stem cells have been isolated from a variety of adult CNS ventricular regions, including the frontal lobe, conus medullaris, thoracic spinal cord, brain stem, and hypothalamus, and proliferated in vitro using the methods detailed herein. In each of these cases, the neural stem cell exhibits self-maintenance and generates a large number of progeny which include neurons, astrocytes and oligodendrocytes.

Normally, the adult mammalian CNS is mitotically quiescent in vivo with the exception of the subependymal region lining the lateral ventricles in the forebrain. This region contains a subpopulation of constitutively proliferating cells with a cell cycle time of 12.7 hours. BrdU and retroviral labeling of the proliferating cells reveal that none of the newly generated cells differentiate into mature neurons or glia nor do they migrate into other CNS regions (Morshead and Van der Kooy, supra).

The continual proliferation and maintenance of a constant number of cells within the subependyma is explained by two mechanisms. The death of one of the daughter cells after each division maintains the proliferating population at a constant number. The constitutively dividing population eventually dies out (and hence is not a stem cell population) however, a subpopulation of relatively quiescent cells within the subependyma is able to repopulate the constitutively dividing population. This stem cell-like mode of maintaining the proliferative subependymal population is analogous to other tissues where cells have a short life span and are repopulated by a subpopulation of relatively quiescent cells referred to as stem cells.

As detailed in Example 27, experiments utilizing retrovirus infection of constituitively proliferating cells in vivo and subsequent β-galactosidase (β-gal) reporter gene expression as a non-diluting marker show that with increasing adult mice survival times (of up to 28 days post retrovirus infection) there is a progressive loss of β-gal positive subependymal cells. Relative to 1 day survival animals, 6 days following retrovirus injection there is a 45% loss of β-gal positive cells and 28 days following retrovirus infection there is a 97% loss. Using nested polymerase chain reaction (PCR) to identify single cells containing retroviral DNA it was determined that the loss of β-gal expressing cells is due to the loss of the retrovirally infected cells through cell death, not due to the turn-off of β-gal expression.

Intraperitoneal injections of BrdU (a thymidine analog that is incorporated into the DNA of dividing cells) reveal that 33% of the cells within some regions of the subependyma make up the normally constituitively dividing population (see Morshead and van der Kooy, *J. Neurosci.* 12:249 (1992)). The number of BrdU labelled cells decreases over time. By 30 days after BrdU labeling, only 3% of the dividing cells are still labelled. The heavy labeling of only a small number of cells 30 days after BrdU injections demonstrates that although the labelled cells were dividing at the time of the injections they were relatively quiescent for the 30 day period. This suggests that these few labeled cells are stem cells rather than cells of the constitutively proliferating population.

The above two examples support the hypothesis that the maintenance of the constant number of proliferating subependymal cells seen throughout adult life requires the presence of a relatively quiescent stem cell that proliferates sporadically to replenish the constitutively proliferating population and to self-renew.

As detailed in Example 24, the constitutively dividing subependymal cells can be killed off by injecting high doses of radioactive thymidine for the duration of the cell cycle at intervals less than S-phase duration. At one day post-kill the proliferating population is 10% of controls and by 8 days the proliferating population is back to control levels. If the

replenished population is due to the recruitment of normally quiescent stem cells into the proliferative mode, then a second kill at the time that stem cells are generating progeny to repopulate the subependyma should alter the number of cells within the constitutively proliferating population. When a second kill is done 2 days after the initial kill, 8 days later the constitutively proliferating population is only 45% of the control values (animals receiving no thymidine kill treatment) or animals that received only one kill at day 0 (the time of the first kill). The reduction in the number of proliferative cells in the subependyma is maintained at 63% even at 31 days after the second kill. When a second kill is done on day 4, the proliferating population returns to 85% of control values 8 days later. These results suggest that the normally quiescent stem cell is recruited into the proliferative mode within the first two days after the initial kill and that by 4 days the stem cell no longer needs to be recruited to repopulate the subependyma.

As detailed in Example 26 below, an experiment was performed to determine whether the in vitro stem cell is derived from the constitutively proliferating population or from the quiescent population. Animals were treated in one of the following ways:

Group 1. Control

High dose of radioactive thymidine were given on:

Group 2. day 0

Group 3. day 0 and day 2

Group 4. day 0 and day 4

16 to 20 following the last injection animals were killed and stem cells isolated from the striatum (including the subependymal region) via the methods described in Example 2 below.

In groups 2–4 the constitutively proliferating population was killed. In group 3 stem cells that are recruited into the cell cycle to repopulate the subependymal proliferating cells were also killed.

Number of Neurospheres produced in vitro:

Group 1. 100% (Control)

Group 2. 100%

Group 3. 45%

Group 4. 85%

These results demonstrate that when you eliminate nearly all of the constitutively proliferating cells in the subependyma this does not affect the number of stem cells that can be isolated and proliferated in vitro (group 1 vs. group 2 and 4). However, when the normally quiescent cells are killed when they are recruited to repopulate the subependyma (as with group 3) the number of stem cells that can be isolated in vitro is significantly reduced (group 3 vs. group 1 and 2). By 4 days after the first kill most of the stem cells themselves are no longer turning over and as a result are not killed by the second series of tritiated thymidine injections (hence, only a 15% reduction [group 4] compared to 55% reduction [group 3]).

The above results demonstrate that, in adult, the stem cells which are proliferated in vitro are derived from the quiescent population of subependymal cells in vivo. This also explains why stem cells can be derived from CNS ventricular regions, other than the forebrain, which do not have a subpopulation of constitutively proliferating cells.

In Vitro Proliferation of Neural Stem Cells

Cells can be obtained from donor tissue by dissociation of individual cells from the connecting extracellular matrix of the tissue. Tissue from a particular neural region is removed from the brain using a sterile procedure, and the cells are dissociated using any method known in the art including treatment with enzymes such as trypsin, collagenase and the like, or by using physical methods of dissociation such as with a blunt instrument. Dissociation of fetal cells can be carried out in tissue culture medium, while a preferable medium for dissociation of juvenile and adult cells is low Ca^{2+} artificial cerebral spinal fluid (aCSF). Regular aCSF contains 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose. Low Ca^{2+} aCSF contains the same ingredients except for $MgCl_2$ at a concentration of 3.2 mM and $CaCl_2$ at a concentration of 0.1 mM. Dissociated cells are centrifuged at low speed, between 200 and 2000 rpm, usually between 400 and 800 rpm, and then resuspended in culture medium. The neural cells can be cultured in suspension or on a fixed substrate. However, substrates tend to induce differentiation of the neural stem cell progeny. Thus, suspension cultures are preferred if large numbers of undifferentiated neural stem cell progeny are desired. Cell suspensions are seeded in any receptacle capable of sustaining cells, particularly culture flasks, culture plates or roller bottles, and more particularly in small culture flasks such as 25 cm^2 culture flasks. Cells cultured in suspension are resuspended at approximately 5×10^4 to 2×10^5 cells/ml, preferably 1×10^5 cells/ml. Cells plated on a fixed substrate are plated at approximately $2–3\times10^3$ cells/cm^2, preferably 2.5×10^3 cells/cm^2.

The dissociated neural cells can be placed into any known culture medium capable of supporting cell growth, including HEM, DMEM, RPMI, F-12, and the like, containing supplements which are required for cellular metabolism such as glutamine and other amino acids, vitamins, minerals and useful proteins such as transferrin and the like. Medium may also contain antibiotics to prevent contamination with yeast, bacteria and fungi such as penicillin, streptomycin, gentamicin and the like. In some cases, the medium may contain serum derived from bovine, equine, chicken and the like. However, a preferred embodiment for proliferation of neural stem cells is to use a defined, serum-free culture medium, as serum tends to induce differentiation and contains unknown components (i.e. is undefined). A defined culture medium is also preferred if the cells are to be used for transplantation purposes. A particularly preferable culture medium is a defined culture medium comprising a mixture of DMEM, F12, and a defined hormone and salt mixture. This culture medium is referred to herein as "Complete Medium" and is described in detail in Example 3.

Conditions for culturing should be close to physiological conditions. The pH of the culture medium should be close to physiological pH, preferably between pH 6–8, more preferably between about pH 7 to 7.8, with pH 7.4 being most preferred. Physiological temperatures range between about 30° C. to 40° C. Cells are preferably cultured at temperatures between about 32° C. to about 38° C., and more preferably between about 35° C. to about 37° C.

The culture medium is supplemented with at least one proliferation-inducing growth factor. As used herein, the term "growth factor" refers to a protein, peptide or other molecule having a growth, proliferative, differentiative, or trophic effect on neural stem cells and/or neural stem cell progeny. Growth factors which may be used for inducing proliferation include any trophic factor that allows neural stem cells and precursor cells to proliferate, including any molecule which binds to a receptor on the surface of the cell to exert a trophic, or growth-inducing effect on the cell. Preferred proliferation-inducing growth factors include EGF, amphiregulin, acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), transforming growth factor alpha (TGFα), and combinations thereof.

Preferred proliferation-inducing growth factors include EGF and TGFα. A preferred combination of proliferation-inducing growth factors is EGF or TGFC with FGF-1 or FGF-2. Growth factors are usually added to the culture medium at concentrations ranging between about 1 fg/ml to 1 mg/ml. Concentrations between about 1 to 100 ng/ml are usually sufficient. Simple titration experiments can be easily performed to determine the optimal concentration of a particular growth factor.

In addition to proliferation-inducing growth factors, other growth factors may be added to the culture medium that influence proliferation and differentiation of the cells including NGF, platelet-derived growth factor (PDGF), thyrotropin releasing hormone (TRH), transforming growth factor betas (TGFβs), insulin-like growth factor (IGF_{-1}) and the like.

Within 3–4 days in the presence of a proliferation-inducing growth factor, a multipotent neural stem cell begins to divide giving rise to a cluster of undifferentiated cells referred to herein as a "neurosphere". The cells of a single neurosphere are clonal in nature because they are the progeny of a single neural stem cell. In the continued presence of a proliferation-inducing growth factor such as EGF or the like, precursor cells within the neurosphere continue to divide resulting in an increase in the size of the neurosphere and the number of undifferentiated cells. The neurosphere is not immunoreactive for GFAP, neurofilament (NF), neuron-specific enolase (NSE) or myelin basic protein (MBP). However, precursor cells within the neurosphere are immunoreactive for nestin, an intermediate filament protein found in many types of undifferentiated CNS cells. The nestin marker was characterized by Lehndahl et al., *Cell* 60:585–595 (1990). Antibodies are available to identify nestin, including the rat antibody referred to as Rat401. The mature phenotypes associated with the differentiated cell types that may be derived from the neural stem cell progeny are predominantly negative for the nestin phenotype.

After about 4 to 5 days in the absence of a substrate, the proliferating neurospheres lift off the floor of the culture dish and tend to form the free-floating clusters characteristic of neurospheres. Floating neurospheres are depicted in FIG. 2*d*. It is possible to vary the culture conditions so that while the precursor cells still express the nestin phenotype, they do not form the characteristic neurospheres. The proliferating precursor cells of the neurosphere continue to proliferate in suspension. After about 3–10 days in vitro, and more particularly after about 6–7 days in vitro, the proliferating neurospheres are fed every 2–7 days, preferably every 2–4 days by gentle centrifugation and resuspension in Complete Medium containing a growth factor.

The neurospheres of the suspension culture can be easily passaged to reinitiate proliferation. After 6–7 days in vitro, the culture flasks are shaken well and the neurospheres allowed to settle on the bottom corner of the flask. The neurospheres are then transferred to a 50 ml centrifuge tube and centrifuged at low speed. The medium is aspirated, and the neurospheres are resuspended in a small amount of Complete Medium. Individual cells in the neurospheres can be separated by physical dissociation of the neurospheres with a blunt instrument, for example, by triturating the neurospheres with a pipette, especially a fire polished pasteur pipette, to form a single cell suspension of neural stem cell progeny. The cells are then counted and replated at the desired density to reinitiate proliferation. Single cells from the dissociated neurospheres are suspended in Complete Medium containing growth factor, and a percentage of these cells proliferate and form new neurospheres largely composed of undifferentiated cells. This procedure can be repeated weekly to result in a logarithmic increase in the number of viable cells at each passage. The procedure is continued until the desired number of precursor cells is obtained.

The number of neural stem cell progeny proliferated in vitro from the mammalian CNS can be increased dramatically by injecting a growth factor or combination of growth factors, for example EGF, FGF, or EGF and FGF together, into the ventricles of the donor in vivo using the in vivo proliferation methods described in more detail below. As detailed in Example 31 below, 6 days after infusion of EGF into the lateral ventricle of a mouse forebrain, the walls of the ventricle were removed and the stem cells harvested. Infusion of EGF into the lateral ventricle increased the efficiency of the yield of stem cells that proliferated to form neurospheres.

This ability to enhance the proliferation of neural stem cells should prove invaluable when stem cells are to be harvested for later transplantation back into a patient, thereby making the initial surgery 1) less traumatic because less tissue would have to be removed and 2) more efficient because a greater yield of stem cells per surgery would proliferate in vitro.

Additionally, the patient's stem cells, once they have proliferated in vitro, could also be genetically modified in vitro using the techniques described below. The in vitro genetic modification may be more desirable in certain circumstances than in vivo genetic modification techniques when more control over the infection with the genetic material is required.

Neural stem cell progeny can be cryopreserved until they are needed by any method known in the art. The cells can be suspended in an isotonic solution, preferably a cell culture medium, containing a particular cryopreservant. Such cryopreservants include dimethyl sulfoxide (DMSO), glycerol and the like. These cryopreservants are used at a concentration of 5–15%, preferably 8–10%. Cells are frozen gradually to a temperature of −10° C. to −150° C., preferably −20° C. to −100° C., and more preferably −70° C. to −80° C.

Differentiation of Neural Stem Cell Progeny

Differentiation of the cells can be induced by any method known in the art which activates the cascade of biological events which lead to growth, which include the liberation of inositol triphosphate and intracellular Ca^{2+}, liberation of diacyl glycerol and the activation of protein kinase C and other cellular kinases, and the like. Treatment with phorbol esters, differentiation-inducing growth factors and other chemical signals can induce differentiation. Differentiation can also be induced by plating the cells on a fixed substrate such as flasks, plates, or coverslips coated with an ionically charged surface such as poly-L-lysine and poly-L-ornithine and the like.

Other substrates may be used to induce differentiation such as collagen, fibronectin, laminin, MATRIGEL™ (Collaborative Research), and the like. Differentiation can also be induced by leaving the cells in suspension in the presence of a proliferation-inducing growth factor, without reinitiation of proliferation (i.e. without dissociating the neurospheres).

A preferred method for inducing differentiation of the neural stem cell progeny comprises culturing the cells on a fixed substrate in a culture medium that is free of the proliferation-inducing growth factor. After removal of the proliferation-inducing growth factor, the cells adhere to the

substrate (e.g. poly-ornithine-treated plastic or glass), flatten, and begin to differentiate into neurons and glial cells. At this stage the culture medium may contain serum such as 0.5–1.0% fetal bovine serum (FBS). However, for certain uses, if defined conditions are required, serum would not be used. Within 2–3 days, most or all of the neural stem cell progeny begin to lose immunoreactivity for nestin and begin to express antigens specific for neurons, astrocytes or oligodendrocytes as determined by immunocytochemistry techniques well known in the art.

Immunocytochemistry (e.g. dual-label immunofluorescence and immunoperoxidase methods) utilizes antibodies that detect cell proteins to distinguish the cellular characteristics or phenotypic properties of neurons from astrocytes and oligodendrocytes. In particular, cellular markers for neurons include NSE, NF, β-tub, MAP-2; and for glia, GFAP (an identifier of astrocytes), galactocerebroside (GalC) (a myelin glycolipid identifier of oligodendrocytes), and the like.

Immunocytochemistry can also be used to detect the expression of neurotransmitters, or in some cases the expression of enzymes responsible for neurotransmitter synthesis. For the identification of neurons, antibodies can be used that detect the presence of acetylcholine (ACh), dopamine, epinephrine, norepinephrine, histamine, serotonin or 5-hydroxytryptamine (5-HT), neuropeptides such as substance P, adrenocorticotrophic hormone, vasopressin or antidiuretic hormone, oxytocin, somatostatin, angiotensin II, neurotensin, and bombesin, hypothalamic releasing hormones such as TRH and luteinizing releasing hormone, gastrointestinal peptides such as vasoactive intestinal peptide (VIP) and cholecystokinin (CCK) and CCK-like peptide, opioid peptides such as endorphins like β-endorphin and enkephalins such as met- and leu-enkephalin, prostaglandins, amino acids such as γ-amino butyric acid (GABA), glycine, glutamate, cysteine, taurine and aspartate and dipeptides such as carnosine. Antibodies to neurotransmitter-synthesizing enzymes can also be used such as glutamic acid decarboxylase (GAD) which is involved in the synthesis of GABA, choline acetyltransferase (ChAT) for ACh synthesis, dopa decarboxylase (DDC) for dopamine, dopamine-β-hydroxylase (DBH) for norepinephrine, and amino acid decarboxylase for 5-HT. Antibodies to enzymes that are involved in the deactivation of neurotransmitters may also be useful such as acetyl cholinesterase (AChE) which deactivates ACh. Antibodies to enzymes involved in the reuptake of neurotransmitters into neuronal terminals such as monoamine oxidase and catechol-o-methyl transferase for dopamine, for 5-HT, and GABA transferase for GABA may also identify neurons. Other markers for neurons include antibodies to neurotransmitter receptors such as the AChE nicotinic and muscarinic receptors, adrenergic receptors α^1, α_2, β^1 and α_2, the dopamine receptor and the like. Cells that contain a high level of melanin, such as those found in the substantia nigra, could be identified using an antibody to melanin.

In situ hybridization histochemistry can also be performed, using cDNA or RNA probes specific for the peptide neurotransmitter or the neurotransmitter synthesizing enzyme mRNAs. These techniques can be combined with immunocytochemical methods to enhance the identification of specific phenotypes. If necessary, the antibodies and molecular probes discussed above can be applied to Western and Northern blot procedures respectively to aid in cell identification.

A preferred method for the identification of neurons uses immunocytochemistry to detect immunoreactivity for NSE, NF, NeuN, and the neuron specific protein, tau-1. Because these markers are highly reliable, they will continue to be useful for the primary identification of neurons, however neurons can also be identified based on their specific neurotransmitter phenotype as previously described.

Type I astrocytes, which are differentiated glial cells that have a flat, protoplasmic/fibroblast-like morphology, are preferably identified by their immunoreactivity for GFAP but not A2B5. Type II astrocytes, which are differentiated glial cells that display a stellate process-bearing morphology, are preferably identified using immunocytochemistry by their phenotype GFAP(+), A2B5(+) phenotype.

Cells that do not express intermediate filaments specific for neurons or for astrocytes, begin to express markers specific for oligodendrocytes in a correct temporal fashion. That is, the cells first become immunoreactive for O4, galactocerebroside (GalC, a myelin glycolipid) and finally, MBP. These cells also possess a characteristic oligodendrocyte morphology.

The present invention provides a method of influencing the relative proportion of these differentiated cell types by the addition of exogenous growth factors during he differentiation stage of the precursor cells. By using dual-label mmunofluorescence and immunoperoxidase methods with various neuronal- and glial-specific antibodies, the effect of the exogenous growth factors on the differentiating cells can be determined.

The biological effects of growth and trophic factors are generally mediated through binding to cell surface receptors. The receptors for a number of these factors have been identified and antibodies and molecular probes for specific receptors are available. Neural stem cells can be analyzed for the presence of growth factor receptors at all stages of differentiation. In many cases, the identification of a particular receptor will define the strategy to use in further differentiating the cells along specific developmental pathways with the addition of exogenous growth or trophic factors.

Exogenous growth factors can be added alone or in various combinations. They can also be added in a temporal sequence (i.e. exposure to a first growth factor influences the expression of a second growth factor receptor, *Neuron* 4:189–201 (1990). Among the growth factors and other molecules that can be used to influence the differentiation of precursor cells in vitro are FGF-1, FGF-2, ciliary neurotrophic factor (CNTF), NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3, neurotrophin 4, interleukins, leukemia inhibitory factor (LIF), cyclic adenosine monophosphate, forskolin, tetanus toxin, high levels of potassium, amphiregulin, TGF-α, TGF-β, insulin-like growth factors, dexamethasone (glucocorticoid hormone), isobutyl 3-methylxanthine, somatostatin, growth hormone, retinoic acid, and PDGF. These and other growth factors and molecules will find use in the present invention.

Genetic Modification of Neural Stem Cell Progeny

Although the precursor cells are non-transformed primary cells, they possess features of a continuous cell line. In the undifferentiated state, in the presence of a proliferation-inducing growth factor such as EGF, the cells continuously divide and are therefore excellent targets for genetic modification. The term "genetic modification" as used herein refers to the stable or transient alteration of the genotype of a precursor cell by intentional introduction of exogenous DNA. DNA may be synthetic, or naturally derived, and may

contain genes, portions of genes, or other useful DNA sequences. The term "genetic modification" as used herein is not meant to include naturally occurring alterations such as that which occurs through natural viral activity, natural genetic recombination, or the like.

Exogenous DNA may be introduced to a precursor cell by viral vectors (retrovirus, modified herpes viral, herpes-viral, adenovirus, adeno-associated virus, and the like) or direct DNA transfection (lipofection, calcium phosphate transfection, DEAE-dextran, electroporation, and the like). The genetically modified cells of the present invention possess the added advantage of having the capacity to fully differentiate to produce neurons or macroglial cells in a reproducible fashion using a number of differentiation protocols.

In another embodiment, the precursor cells are derived from transgenic animals, and thus are in a sense already genetically modified. There are several methods presently used for generating transgenic animals. The technique used most often is direct miicroinjection of DNA into single-celled fertilized eggs. Other techniques include retroviral-mediated transfer, or gene transfer in embryonic stem cells. These techniques and others are detailed by Hogan et al. in *Manipulating the Mouse Embryo, A Laboratory Manual* (Cold Spring Harbor Laboratory Ed., 1986). Use of these transgenic animals has certain advantages including the fact that there is no need to transfect healthy neurospheres. Precursor cells derived from transgenic animals will exhibit stable gene expression. Using transgenic animals, it is possible to breed in new genetic combinations. The transgenic animal may have integrated into its genome any useful gene that is expressed by neural cells. Examples of useful DNA are given below in the discussion of genetically modifying precursor cells.

A significant challenge for cellular transplantation in the CNS is the identification of the donor cells after implantation within the host. A number of strategies have been employed to mark donor cells, including tritiated labels, fluorescent dyes, dextrans, and viral vectors carrying reporter genes. However, these methods suffer from inherent problems of toxicity, stability, or dilution over the long term. The use of neural cells derived from transgenic animals may provide an improved means by which identification of transplanted neural cells can be achieved. A transgenic marking system provides a more stable and efficient method for cell labeling. In this system, promoter elements, for example for GFAP and MBP, can direct the expression of the *E. coli* β-galactosidase reporter gene in transgenic mice. In these systems, cell-specific expression of the reporter gene occurs in astrocytes (GFAP-lacZ) and in oligodendrocytes (MBP-lacZ) in a developmentally-regulated manner. The Rosa26 transgenic mice, described in Example 45, is one example of a transgenic marking system in which all cells ubiquitously express β-galactosidase.

Once propagated, the neurosphere cells are mechanically dissociated into a single cell suspension and plated on petri dishes in a medium where they are allowed to attach overnight. The precursor cells are then genetically modified. If the precursor cells are generated from transgenic animals, then they may or may not be subjected to further genetic modification, depending upon the properties desired of the cells. Any useful genetic modification of the cells is within the scope of the present invention. For example, precursor cells may be modified to produce or increase production of a biologically active substance such as a neurotransmitter or growth factor or the like. The genetic modification is performed either by infection with recombinant retroviruses or transfection using methods known in the art (see Maniatis et al., in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. (1982)). Briefly, the chimeric gene constructs will contain viral, for example retroviral long terminal repeat (LTR), simian virus 40 (SV40), cytomegalovirus (CMV); or mammalian cell-specific promoters such as tyrosine hydroxylase (TH, a marker for dopamine cells), DBH, phenylethanolamine N-methyltransferase (PNMT), ChAT, GFAP, NSE, the NF proteins (NF-L, NF-M, NF-H, and the like) that direct the expression of the structural genes encoding the desired protein. In addition, the vectors will include a drug selection marker, such as the *E. coli* aminoglycoside phosphotransferase gene, which when coinfected with the experimental gene confers resistance to geneticin (G418), a protein synthesis inhibitor.

When the genetic modification is for the production of a biologically active substance, the substance will generally be one that is useful for the treatment of a given CNS disorder. For example, it may be desired to genetically modify cells so they secrete a certain growth factor product. As used herein, the term "growth factor product" refers to a protein, peptide, mitogen, or other molecule having a growth, proliferative, differentiative, or trophic effect. Growth factor products useful in the treatment of CNS disorders include, but are not limited to, NGF, BDNF, the neurotrophins (NT-3, NT-4/NT-5), CNTF, amphiregulin, FGF-1, FGF-2, EGF, TGFα, TGFβs, PDGF, IGFs, and the interleukins.

Cells can also be modified to express a certain growth factor receptor (r) including, but not limited to, p75 low affinity NGFr, CNTFr, the trk family of neurotrophin receptors (trk, trkB, trkC), EGFr, FGFr, and amphiregulin receptors. Cells can be engineered to produce various neurotransmitters or their receptors such as serotonin, L-dopa, dopamine, norepinephrine, epinephrine, tachykinin, substance-P, endorphin, enkephalin, histamine, N-methyl D-aspartate, glycine, glutamate, GABA, ACh, and the like. Useful neurotransmitter-synthesizing genes include TH, DDC, DBH, PNMT, GAD, tryptophan hydroxylase, ChAT, and histidine decarboxylase. Genes that encode for various neuropeptides, which may prove useful in the treatment of CNS disorders, include substance-P, neuropeptide-Y, enkephalin, vasopressin, VIP, glucagon, bombesin, CCK, somatostatin, calcitonin gene-related peptide, and the like.

After successfully transfected/infected cells are selected they can be cloned using limiting dilution in 96 multi-well plates and assayed for the presence of the desired biologically active substance. Clones that express high levels of the desired substance are grown and their numbers expanded in T-flasks. The specific cell line can then be cyropreserved. Multiple clones of genetically modified precursor cells will be obtained. Some may give rise preferentially to neuronal cells, and some to glial cells.

The genetically modified precursor cells can be implanted for cell/gene therapy into the CNS of a recipient in need of the biologically active molecule produced by the genetically modified cells. Transplantation techniques are detailed below.

Alternatively, the genetically modified precursor cells can be subjected to various differentiation protocols in vitro prior to implantation. For example, genetically modified precursor cells may be removed from the culture medium which allows proliferation and differentiated using any of the protocols described above. The protocol used will depend upon the type of genetically modified cell desired.

Once the cells have differentiated, they are again assayed for expression of the desired protein. Cells having the desired phenotype can be isolated and implanted into recipients in need of the protein or biologically active molecule that is expressed by the genetically modified cell.

Transplantation of Neural Stem Cell Progeny Alleviate Disorders of the CNS in Animal Models Caused by Disease or Injury

It is well recognized in the art that transplantation of tissue into the CNS offers the potential for treatment of neurodegenerative disorders and CNS damage due to injury (review: Lindvall, (1991) *Tins* vol. 14(8): 376–383). Transplantation of new cells into the damaged CNS has the potential to repair damaged circuitries and provide neurotransmitters thereby restoring neurological function. However, the absence of suitable cells for transplantation purposes has prevented the full potential of this procedure from being met. "Suitable" cells are cells that meet the following criteria: 1) can be obtained in large numbers; 2) can be proliferated in vitro to allow insertion of genetic material, if necessary; 3) capable of surviving indefinitely but stop growing after transplantation to the brain; 4) are nonimmunogenic, preferably obtained from a patient's own tissue; 5) are able to form normal neural connections and respond to neural physiological signals (Bjorklund (1991) *TINS* Vol. 14(8): 319–322). The progeny of multipotent neural stem cells obtainable from embryonic or adult CNS tissue, which are able to divide indefinitely when maintained in vitro using the culture conditions described herein, meet all of the desirable requirements of cells suitable for neural transplantation purposes and are a particularly suitable cell line as the cells have not been immortalized and are not of tumorigenic origin. The use of multipotent neural stem cells in the treatment of neurological disorders and CNS damage can be demonstrated by the use of animal models.

The neural stem cell progeny can be administered to any animal with abnormal neurological or neurodegenerative symptoms obtained in any manner, including those obtained as a result of mechanical, chemical, or electrolytic lesions, as a result of experimental aspiration of neural areas, or as a result of aging processes. Particularly preferable lesions in non-human animal models are obtained with 6-hydroxy-dopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), ibotenic acid and the like.

The instant invention allows the use of precursor cells prepared from donor tissue which is xenogeneic to the host. Since the CNS is a somewhat immunoprivileged site, the immune response is significantly less to xenografts, than elsewhere in the body. In general, however, in order for xenografts to be successful it is preferred that some method of reducing or eliminating the immune response to the implanted tissue be employed. Thus recipients will often be immunosuppressed, either through the use of immunosuppressive drugs such as cyclosporin, or through local immunosuppression strategies employing locally applied immunosuppressants. Local immunosuppression is disclosed by Gruber, *Transplantation* 54:1–11 (1992). Rossini, U.S. Pat. No. 5,026,365, discloses encapsulation methods suitable for local immunosuppression.

As an alternative to employing immunosuppression techniques, methods of gene replacement or knockout using homologous recombination in embryonic stem cells, taught by Smithies et al. (*Nature*, 317:230–234 (1985), and extended to gene replacement or knockout in cell lines (H. Zheng 35 al., *PNAS*, 88:8067–8071 (1991)), can be applied to precursor cells for the ablation of major histocompatibility complex (MHC) genes. Precursor cells lacking MHC expression would allow for the grafting of enriched neural cell populations across allogeneic, and perhaps even xenogeneic, histocompatibility barriers without the need to immunosuppress the recipient. General reviews and citations for the use of recombinant methods to reduce antigenicity of donor cells are also disclosed by Gruber (supra). Exemplary approaches to the reduction of immunogenicity of transplants by surface modification are disclosed by Faustman WO 92/04033 (1992). Alternatively the immunogenicity of the graft may be reduced by preparing precursor cells from a transgenic animal that has altered or deleted MHC antigens.

Grafting of precursor cells prepared from tissue which is allogeneic to that of the recipient will most often employ tissue typing in an effort to most closely match the histocompatibility type of the recipient. Donor cell age as well as age of the recipient have been demonstrated to be important factors in improving the probability of neuronal graft survival. The efficiency of grafting is reduced with increased age of donor cells. Furthermore, grafts are more readily accepted by younger recipients compared to older recipients. These two factors are likely to be as important for glial graft survival as they are for neuronal graft survival.

In some instances, it may be possible to prepare neural stem cell progeny from the recipient's own nervous system (e.g.in the case of tumor removal biopsies etc,).

In such instances the neural stem cell progeny may be generated from dissociated tissue and proliferated in vitro using the methods described above. Upon suitable expansion of cell numbers, the precursor cells may be harvested, genetically modified if necessary, and readied for direct injection into the recipient's CNS.

Transplantation can be done bilaterally, or, in the case of a patient suffering from Parkinson's Disease, contralateral to the most affected side. Surgery is performed in a manner in which particular brain regions may be located, such as in relation to skull sutures, particularly with a stereotaxic guide. Cells are delivered throughout any affected neural area, in particular to the basal ganglia, and preferably to the caudate and putamen, the nucleus basalis or the substantia nigra. Cells are administered to the particular region using any method which maintains the integrity of surrounding areas of the brain, preferably by injection cannula. Injection methods exemplified by those used by Duncan et al. *J.Neurocytology*, 17:351–361 (1988), and scaled up and modified for use in humans are preferred. Methods taught by Gage et al., supra, for the injection of cell suspensions such as fibroblasts into the CNS may also be employed for injection of neural precursor cells. Additional approaches and methods may be found in *Neural Grafting in the Mammalian CNS*, Bjorklund and Stenevi, eds., (1985).

Although solid tissue fragments and cell suspensions of neural tissue are immunogenic as a whole, it could be possible that individual cell types within the graft are themselves immunogenic to a lesser degree. For example, Bartlett et al. (*Prog. Brain Res.* 82: 153–160 (1990)) have abrogated neural allograft rejection by pre-selecting a subpopulation of embryonic neuroepithelial cells for grafting by the use of immunobead separation on the basis of MHC expression. Thus, another approach is provided to reduce the chances of allo and xenograft rejection by the recipient without the use of immunosuppression techniques.

Neural stem cell progeny when administered to the particular neural region preferably form a neural graft, wherein

the neuronal cells form normal neuronal or synaptic connections with neighboring neurons, and maintain contact with transplanted or existing glial cells which may form myelin sheaths around the neurons' axons, and provide a trophic influence for the neurons. As these transplanted cells form connections, they re-establish the neuronal networks which have been damaged due to disease and aging.

Survival of the graft in the living host can be examined using various non-invasive scans such as computerized axial tomography (CAT scan or CT scan), nuclear magnetic resonance or magnetic resonance imaging (NMR or MRI) or more preferably positron emission tomography (PET) scans. Post-mortem examination of graft survival can be done by removing the neural tissue, and examining the affected region macroscopically, or more preferably using microscopy. Cells can be stained with any stains visible under light or electron microscopic conditions, more particularly with stains which are specific for neurons and glia. Particularly useful are monoclonal antibodies which identify neuronal cell surface markers such as the M6 antibody which identifies mouse neurons. Most preferable are antibodies which identify any neurotransmitters, particularly those directed to GABA, TH, ChAT, and substance P, and to enzymes involved in the synthesis of neurotransmitters, in particular, GAD. Transplanted cells can also be identified by prior incorporation of tracer dyes such as rhodamine- or fluorescein-labelled microspheres, fast blue, bisbenzamide or retrovirally introduced histochemical markers such as the lac Z gene which produces beta galactosidase.

Functional integration of the graft into the host's neural tissue can be assessed by examining the effectiveness of grafts on restoring various functions, including but not limited to tests for endocrine, motor, cognitive and sensory functions. Motor tests which can be used include those which quantitate rotational movement away from the degenerated side of the brain, and those which quantitate slowness of movement, balance, coordination, akinesia or lack of movement, rigidity and tremors. Cognitive tests include various tests of ability to perform everyday tasks, as well as various memory tests, including maze performance.

Neural stem cell progeny can be produced and transplanted using the above procedures to treat demyelination diseases. Human demyelinating diseases for which the cells of the present invention may provide treatment include disseminated perivenous encephalomyelitis, MS (Charcot and Marburg types), neuromyelitis optica, concentric sclerosis, acute, disseminated encephalomyelitides, post encephalomyelitis, postvaccinal encephalomyelitis, acute hemorrhagic leukoencephalopathy, progressive multifocal leukoencephalopathy, idiopathic polyneuritis, diphtheric neuropathy, Pelizaeus-Merzbacher disease, neuromyelitis optica, diffuse cerebral sclerosis, central pontine myelinosis, spongiform leukodystrophy, and leukodystrophy (Alexander type).

Areas of demyelination in humans is generally associated with plaque like structures. Plaques can be visualized by magnetic resonance imaging. Accessible plaques are the target area for injection of neural stem cell progeny. Standard stereotactic neurosurgical methods are used to inject cell suspensions both into the brain and spinal cord. Generally, the cells can be obtained from any of the sources discussed above. However, in the case of demyelinating diseases with a genetic basis directly affecting the ability of the myelin forming cell to myelinate axons, allogeneic tissue would be a preferred source of the cells as autologous tissue (i.e. the recipient's cells) would generally not be useful unless the cells have been modified in some way to insure the lesion will not continue (e.g. genetically modifying the cells to cure the demyelination lesion).

Oligodendrocytes derived from neural stem cell progeny proliferated and differentiated in vitro may be injected into demyelinated target areas in the recipient. Appropriate amounts of type I astrocytes may also be injected. Type I astrocytes are known to secrete PDGF which promotes both migration and cell division of oligodendrocytes. [Nobel et al., *Nature* 333:560–652 (1988); Richardson et al., *Cell*, 53:309–319 (1988)].

A preferred treatment of demyelination disease uses undifferentiated neural stem cell progeny. Neurospheres grown in the presence of a proliferation-inducing growth factor such as EGF can be dissociated to obtain individual precursor cells which are then placed in injection medium and injected directly into the demyelinated target region. The cells differentiate in vivo. Astrocytes can promote remyelination in various paradigms. Therefore, in instances where oligodendrocyte proliferation is important, the ability of precursor cells to give rise to type I astrocytes may be useful. In other situations, PDGF may be applied topically during the transplantation as well as with repeated doses to the implant site thereafter.

The injection of neural stem cell progeny in remyelination therapy provides, amongst other types of cells, a source of immature type I astrocytes at the implant site. This is a significant feature because immature astrocytes (as opposed to mature astrocytes) have a number of specific characteristics that make them particularly suited for remyelination therapy. First, immature, as opposed to mature, type I astrocytes are known to migrate away from the implant site [Lindsay et. al, *Neurosci.* 12:513–530 (1984)] when implanted into a mature recipient and become associated with blood vessels in the recipient's CNS [Silver et al., WO 91/06631 (1991)]. This is at least partially due to the fact that immature astrocytes are intrinsically more motile than mature astrocytes. [Duffy et al., *Exp Cell Res.* 139:145–157 (1982), Table VII]. Type I astrocytes differentiating at or near the precursor cell implant site should have maximal motility and thereby optimize the opportunity for oligodendrocyte growth and division at sites distant from the implant. The localization of the astrocytes near blood vessels is also significant from a therapeutic standpoint since (at least in MS) most plaques have a close anatomical relationship with one or more veins.

Another characteristic of immature astrocytes that makes them particularly suited for remyelination therapy is that they undergo a lesser degree of cell death than mature type I astrocytes. (Silver et al., supra)

Any suitable method for the implantation of precursor cells near to the demyelinated targets may be used so that the cells can become associated with the demyelinated axons. Glial cells are motile and are known to migrate to, along, and across their neuronal targets thereby allowing the spacing of injections. Remyelination by the injection of precursor cells is a useful therapeutic in a wide range of demyelinating conditions. It should also be borne in mind that in some circumstances remyelination by precursor cells will not result in permanent remyelination, and repeated injections will be required. Such therapeutic approaches offer advantage over leaving the condition untreated and may spare the recipient's life.

In Vivo Proliferation, Differentiation, and Genetic Modification of Neural Stem Cell Progeny

Neural stem cells and their progeny can be induced to proliferate and differentiate in vivo by administering to the

host, any growth factor(s) or pharmaceutical composition that will induce proliferation and differentiation of the cells. These growth factors include any growth factor known in the art, including the growth factors described above for in vitro proliferation and differentiation. Pharmaceutical compositions include any substance that blocks the inhibitory influence and/or stimulates neural stem cells and stem cell progeny to proliferate and ultimately differentiate. Thus, the techniques described above to proliferate, differentiate, and genetically modify neural stem cells in vitro can be adapted to in vivo techniques, to achieve similar results. Such in vivo manipulation and modification of these cells allows cells lost, due to injury or disease, to be endogenously replaced, thus obviating the need for transplanting foreign cells into a patient. Additionally, the cells can be modified or genetically engineered in vivo so that they express various biological agents useful in the treatment of neurological disorders. Administration of growth factors can be done by any method, including injection cannula, transfection of cells with growth hormone-expressing vectors, injection, timed-release apparati which can administer substances at the desired site, and the like. Pharmaceutical compositions can be administered by any method, including injection cannula, injection, oral administration, timed-release apparati and the like. The neural stem cells can be induced to proliferate and differentiate in vivo by induction with particular growth factors or pharmaceutical compositions which will induce their proliferation and differentiation. Therefore, this latter method circumvents the problems associated with transplantation and immune reactions to foreign cells. Any growth factor can be used, particularly EGF, TGFα, FGF-1, FGF-2 and NGF.

Growth factors can be administered in any manner known in the art in which the factors may either pass through or by-pass the blood-brain barrier. Methods for allowing factors to pass through the blood-brain barrier include minimizing the size of the factor, or providing hydrophobic factors which may pass through more easily.

The fact that neural stem cells are located in the tissues lining ventricles of mature brains offers several advantages for the modification and manipulation of these cells in vivo and the ultimate treatment of various neurological diseases, disorders, and injury that affect different regions of the CNS. Therapy for these can be tailored accordingly so that stem cells surrounding ventricles near the affected region would be manipulated or modified in vivo using the methods described herein. The ventricular system is found in nearly all brain regions and thus allows easier access to the affected areas. If one wants to modify the stem cells in vivo by exposing them to a composition comprising a growth factor or a viral vector, it is relatively easy to implant a device that administers the composition to the ventricle and thus, to the neural stem cells. For example, a cannula attached to an osmotic pump may be used to deliver the composition. Alternatively, the composition may be injected directly into the ventricles. The neural stem cell progeny can migrate into regions that have been damaged as a result of injury or disease. Furthermore, the close proximity of the ventricles to many brain regions would allow for the diffusion of a secreted neurological agent by the stem cells or their progeny.

For treatment of Huntington's Disease, Alzheimer's Disease, Parkinson's Disease, and other neurological disorders affecting primarily the forebrain, growth factors or other neurological agents would be delivered to the ventricles of the forebrain to affect in vivo modification or manipulation of the stem cells. For example, Parkinson's Disease is the result of low levels of dopamine in the brain, particularly the striatum. It would be advantageous to induce a patient's own quiescent stem cells to begin to divide in vivo and to induce the progeny of these cells to differentiate into dopaminergic cells in the affected region of the striatum, thus locally raising the levels of dopamine.

Normally the cell bodies of dopaminergic neurons are located in the substantia nigra and adjacent regions of the mesencephalon, with the axons projecting to the striatum. Prior art methods for treating Parkinson's disease usually involves the use of the drug L-Dopa, to raise dopamine levels in the striatum. However, there are disadvantages with this treatment including drug tolerance and side effects. Also, embryonic tissues that produce dopamine have been transplanted into the striatum of human Parkinsonian patients with reasonable success. However, the use of large quantities of fetal human tissue required for this procedure raises serious ethical concerns and practical issues.

The methods and compositions of the present invention provide an alternative to the use of drugs and the controversial use of large quantities of embryonic tissue for treatment of Parkinson's disease. Dopamine cells can be generated in the striatum by the administration of a composition comprising growth factors to the lateral ventricle. A particularly preferred composition comprises a combination of EGF, FGF-2, and heparan sulphate. The composition preferably also comprises serum. After administration of this composition, there is a significant increase in the transcription of messenger RNA (mRNA) for TH in the subventricular region of the striatum, an area which normally does not contain dopaminergic cell bodies. These methods and results are described in detail in Example 34. As detailed in

Example 35, the use of dual labeling tissue to show the distribution of BrdU+ and TH+ cells indicates that, in response to the in vivo administration of growth factors, TH+ cell bodies occur in striatal tissue. Many of these newly generated TH+ cells are also BrdU+.

For the treatment of MS and other demyelinating or hypomyelinating disorders, and for the treatment of Amyotrophic Lateral Sclerosis or other motor neuron diseases, growth factors or other neurological agents would be delivered to the central canal.

In addition to treating CNS tissue immediately surrounding a ventricle, a viral vector, DNA, growth factor, or other neurological agent can be easily administered to the lumbar cistern for circulation throughout the CNS.

Under normal conditions subependymal precursors do not differentiate or migrate, rather, their fate appears to be cell death after an undefined number of cell divisions (Morshead and Van der Kooy, supra). This explanation is also supported by PCR evidence, as described above. Injection of growth factors into the lateral ventricle alters this fate. As described in more detail in Example 27 below, retroviruses were injected into the lateral ventricles for six consecutive days. Implanting cannulae attached to EGF-filled osmotic pumps into the lateral ventricles on the same day as (and 1 or 6 days following) retrovirus injection results in an increase in the total number of RV-β-gal labelled cells 6 days later (from an average of 20 cells/brain to 150 cells/brain).

It is known from the PCR experiments described above that 6 days following retroviral injection no cells exist that contain non-expressed retroviral DNA. Thus these results indicate that the EGF-induced increase in β-gal positive cell number is due to the expansion of the clone size of the retrovirally labelled constitutively proliferative population. It is also possible that part of this increase is due to the activation by EGF of a relatively quiescent stem cell.

Interestingly, this expansion of the number of β-gal labelled cells is accompanied by the migration of these cells away from the subependymal medially, laterally, rostrally, and caudally with subsequent differentiation. Thus, infusion of EGF or similar growth factors induces the proliferation, migration and differentiation of neural stem cells and progenitor cells in vivo, and can be used therapeutically to replace neural cells lost due to injury or disease. In a preferred embodiment EGF and FGF are administered together or sequentially.

The normal fate of the constitutively proliferating cell population (i.e. cell death) can be altered by administering Bcl-2 or genetically modifying the cells with the bcl-2 gene. The gene product is known to prevent programmed cell death (apoptosis) in a variety of cell types. Similar to the EGF experiments, a clonal expansion of the constitutively proliferating cell population is achieved following infection with bcl-2.

Other ways of passing the blood-brain barrier include in vivo transfection of neural stem cells and stem cell progeny with expression vectors containing genes that code for growth factors, so that the cells themselves produce the factor. Any useful genetic modification of the cells is within the scope of the present invention. For example, in addition to genetic modification of the cells to express growth factors, the cells may be modified to express other types of neurological agents such as neurotransmitters. Preferably, the genetic modification is performed either by infection of the cells lining ventricular regions with recombinant retroviruses or transfection using methods known in the art including $CaPO_4$ transfection, DEAE-dextran transfection, polybrene transfection, by protoplast fusion, electroporation, lipofection, and the like [see Maniatis et al., supra]. Any method of genetic modification, now known or later developed can be used. With direct DNA transfection, cells could be modified by particle bombardment, receptor mediated delivery, and cationic liposomes. When chimeric gene constructs are used, they generally will contain viral, for example retroviral long terminal repeat (LTR), simian virus 40 (SV40), cytomegalovirus (CMV); or mammalian cell-specific promoters such as those for TH, DBH, phenylethanolamine N-methyltransferase, ChAT, GFAP, NSE, the NF proteins (NF-L, NF-M, NF-H, and the like) that direct the expression of the structural genes encoding the desired protein.

If a retroviral construct is to be used to genetically modify normally quiescent stem cells, then it is preferable to induce the proliferation of these cells using the methods described herein. For example, an osmotic infusion pump could be used to deliver growth factors to the central canal several days prior to infection with the retrovirus. This assures that there will be actively dividing neural stem cells which are susceptible to infection with the retrovirus.

When the genetic modification is for the production of a biologically active substance, the substance will generally be one that is useful for the treatment of a given CNS disorder. For example, it may be desired to genetically modify cells so they secrete a certain growth factor product. Growth factor products useful in the treatment of CNS disorders are listed above. Cells can also be modified in vivo to express a growth factor receptors, neurotransmitters or their receptors, neurotransmitter-synthesizing genes, neuropeptides, and the like, as discussed above.

Any expression vector known in the art can be used to express the growth factor, as long as it has a promoter which is active in the cell, and appropriate termination and polyadenylation signals. These expression vectors include recombinant vaccinia virus vectors including pSC11, or vectors derived various viruses such as from Simian Virus 40 (SV40, i.e. pSV2-dhfr, pSV2neo, pko-neo, pSV2gpt, pSVT7 and pBABY), from Rous Sarcoma Virus (RSV, i.e. pRSVneo), from mouse mammary tumor virus (MMTV, i.e. pMSG), from adenovirus(pMT2), from herpes simplex virus (HSV, i.e. pTK2 and pHyg), from bovine papillomavirus (BPV, i.e. pdBPV and pBV-1MTHA), from Epstein-Barr Virus (EBV, i.e. p205 and pHEBo) or any other eukaryotic expression vector known in the art.

Other methods for providing growth factors to the area of transplantation include the implantation into the brain in proximity to the graft of any device which can provide an infusion of the factor to the surrounding cells.

In Vitro Models of CNS Development, Function and Dysfunction, and Methods for Screening Effects of Drugs on Neural Cells

Neural stem cell progeny cultured in vitro can be used for the screening of potential neurologically therapeutic compositions. These compositions can be applied to cells in culture at varying dosages, and the response of the cells monitored for various time periods. Physical characteristics of the cells can be analyzed by observing cell and neurite growth with microscopy. The induction of expression of new or increased levels of proteins such as enzymes, receptors and other cell surface molecules, or of neurotransmitters, amino acids, neuropeptides and biogenic amines can be analyzed with any technique known in the art which can identify the alteration of the level of such molecules. These techniques include immunohistochemistry using antibodies against such molecules, or biochemical analysis. Such biochemical analysis includes protein assays, enzymatic assays, receptor binding assays, enzyme-linked immunosorbant assays (ELISA), electrophoretic analysis, analysis with high performance liquid chromatography (HPLC), Western blots, and radioimmune assays (RIA). Nucleic acid analysis such as Northern blots can be used to examine the levels of mRNA coding for these molecules, or for enzymes which synthesize these molecules.

Alternatively, cells treated with these pharmaceutical compositions can be transplanted into an animal, and their survival, ability to form neuronal connections, and biochemical and immunological characteristics examined as previously described.

For the preparation of CNS models, neural stem cells and stem cell progeny are proliferated using the methods described above. Upon removal of the proliferation-inducing growth factor, proliferation of multipotent neural stem cells ceases. The neurospheres can be differentiated using the methods described above, for example by adhering the neurospheres to a substrate such as poly-ornithinetreated plastic or glass where the precursor cells begin to differentiate into neurons and glial cells. Thus, the proliferation-inducing growth factor acts as an extrinsic signaling molecule that can be added or removed at will to control the extent of proliferation.

When the proliferation-inducing growth factor is removed, the growth-factor responsive stem cell progeny can be co-cultured on a feeder layer. Many types of feeder layers may be used, such as fibroblasts, neurons, astrocytes, oligodendrocytes, tumor cell lines, genetically altered cell lines or any cells or substrate with bioactive properties. The feeder layer generally produces a broader range of phenotypes. In this instance, the feeder layer acts as a substrate and

source of both membrane bound and soluble factors that induce and alter the differentiation of the stem cell-generated progeny. Compared to a more inert substance, such as poly-L-ornithine, an astrocyte feeder layer, for example, induces a broader range of neuronal phenotypes as determined by indirect immunocytochemistry at 7 DIV. When differentiated on a poly-L-ornithine coated substrate with 1% FBS, neuronal phenotypes are almost exclusively GABAergic or substance P-ergic. When differentiated on an astrocyte feeder layer, in addition to GABAergic and substance P-ergic neurons, somatostatin, neuropeptide Y (NPY), glutamate and met-enkephalin-containing neurons are present. The astrocytes can be derived from tissue obtained from various brain regions such as the striatum, cortex and spinal cord.

Once the growth factor is removed, the culture medium may contain serum such as 0.5–1.0% FBS. Serum tends to support the differentiation process and enhance cell survival, especially when the differentiating cells are grown at a low density. However, it is possible to culture and differentiate the cells using defined conditions.

Within 1–3 days after removal of the growth factor and placing of the cell in conditions that support differentiation and survival, most or all of the precursor cells begin to lose immunoreactivity for nestin and begin to express antigens specific for neurons, astrocytes or oligodendrocytes. The identification of neurons is confirmed using immunoreactivity for the neuron-specific markers previously mentioned.

The precursor cells described above can be used in methods of determining the effect of a biological agents on neural cells. The term "biological agent" refers to any agent, such as a virus, protein, peptide, amino acid, lipid, carbohydrate, nucleic acid, nucleotide, drug, pro-drug or other substance that may have an effect on neural cells whether such effect is harmful, beneficial, or otherwise. Biological agents that are beneficial to neural cells are referred to herein as "neurological agents", a term which encompasses any biologically or pharmaceutically active substance that may prove potentially useful for the proliferation, differentiation or functioning of CNS cells or treatment of neurological disease or disorder. For example, the term may encompass certain neurotransmitters, neurotransmitter receptors, growth factors, growth factor receptors, and the like, as well as enzymes used in the synthesis of these agents.

Examples of biological agents include growth factors such as FGF-1, FGF-2, EGF and EGF-like ligands, TGFα, IGF-1, NGF, PDGF, and TGFβs; trophic factors such as BDNF, CNTF, and glial-derived neurotrophic factor (GDNF); regulators of intracellular pathways associated with growth factor activity such as phorbol 12-myristate 13-acetate, staurosporine, CGP-41251, tyrphostin, and the like; hormones such as activin and TRH; various proteins and polypeptides such as interleukins, the Bcl-2 gene product, bone morphogenic protein (BMP-2), macrophage inflammatory proteins (MIP-1α, MIP-1β and MIP-2); oligonucleotides such as antisense strands directed, for example, against transcripts for EGF receptors, FGF receptors, and the like; heparin-like molecules such as heparan sulfate; and a variety of other molecules that have an effect on neural stem cells or stem cell progeny including amphiregulin, retinoic acid, and tumor necrosis factor alpha (TNFα).

To determine the effect of a potential biological agent on neural cells, a culture of precursor cells derived from multipotent stem cells can be obtained from normal neural tissue or, alternatively, from a host afflicted with a CNS disease or disorder such as Alzheimer's Disease, Parkinson's Disease, or Down's Syndrome. The choice of culture will depend upon the particular agent being tested and the effects one wishes to achieve. Once the cells are obtained from the desired donor tissue, they are proliferated in vitro in the presence of a proliferation-inducing growth factor.

The ability of various biological agents to increase, decrease or modify in some other way the number and nature of the stem cell progeny proliferated in the presence of EGF or other proliferative factor can be screened on cells proliferated by the methods described in Examples 1–6. For example, it is possible to screen for biological agents that increase the proliferative ability of progenitor cells which would be useful for generating large numbers of cells for transplantation purposes. It is also possible to screen for biological agents which inhibit precursor cell proliferation. In these studies precursor cells are plated in the presence of the biological factor(s) of interest and assayed for the degree of proliferation which occurs. The effects of a biological agent or combination of biological agents on the differentiation and survival of progenitor cells and their progeny can be determined. It is possible to screen neural cells which have already been induced to differentiate prior to the screening. It is also possible to determine the effects of the biological agents on the differentiation process by applying them to precursor cells prior to differentiation. Generally, the biological agent will be solubilized and added to the culture medium at varying concentrations to determine the effect of the agent at each dose. The culture medium may be replenished with the biological agent every couple of days in amounts so as to keep the concentration of the agent somewhat constant.

Changes in proliferation are observed by an increase or decrease in the number of neurospheres that form and/or an increase or decrease in the size of the neurospheres (which is a reflection of the rate of proliferation—determined by the numbers of precursor cells per neurosphere). Thus, the term "regulatory factor" is used herein to refer to a biological factor that has a regulatory effect on the proliferation of stem cells and/or precursor cells. For example, a biological factor would be considered a "regulatory factor" if it increases or decreases the number of stem cells that proliferate in vitro in response to a proliferation-inducing growth factor (such as EGF). Alternatively, the number of stem cells that respond to proliferation-inducing factors may remain the same, but addition of the regulatory factor affects the rate at which the stem cell and stem cell progeny proliferate. A proliferative factor may act as a regulatory factor when used in combination with another proliferative factor. For example, the neurospheres that form in the presence of a combination of bFGF and EGF are significantly larger than the neurospheres that form in the presence of bFGF alone, indicating that the rate of proliferation of stem cells and stem cell progeny is higher.

Other examples of regulatory factors include heparan sulfate, TGFBs, activin, BMP-2, CNTF, retinoic acid, TNFα, MIP-1α, MIP-1β, MIP-2, NGF, PDGF, interleukins, and the Bcl-2 gene product. Antisense molecules that bind to transcripts of proliferative factors and the transcripts for their receptors also regulate stem cell proliferation. Other factors having a regulatory effect on stem cell proliferation include those that interfere with the activation of the c-fos pathway (an intermediate early gene, known to be activated by EGF), including phorbol 12 myristate 13-acetate (PMA; Sigma), which up-regulates the c-fos pathway and staurosporine (Research Biochemical International) and CGP-

41251 (Ciba-Geigy), which down regulate c-fos expression and factors, such as tyrphostin [Fallon, D et al., *Mol. Cell Biol.*, 11 (5): 2697–2703 (1991)] and the like, which suppress tyrosine kinase activation induced by the binding of EGF to its receptor.

Preferred regulatory factors for increasing the rate at which neural stem cell progeny proliferate in response to FGF are heparan sulfate and EGF. Preferred regulatory factors for decreasing the number of stem cells that respond to proliferative factors are members of the TGFβ family, interleukins, MIPs, PDGF, TNFα, retinoic acid (10^{-6}M) and CNTF. Preferred factors for decreasing the size of neurospheres generated by the proliferative factors are members of the TGFβ family, retinoic acid (10^{-6}M) and CNTF.

The regulatory factors are added to the culture medium at a concentration in the range of about 10 pg/ml to 500 ng/ml, preferably about 1 ng/ml to 100 ng/ml. The most preferred concentration for regulatory factors is about 10 ng/ml. The regulatory factor retinoic acid is prepared from a 1 mM stock solution and used at a final concentration between about 0.01 μM and 100 μM, preferably between about 0.05 to 5 μM. Preferred for reducing the proliferative effects of EGF or bFGF on neurosphere generation is a concentration of about 1 μM of retinoic acid. Antisense strands, can be used at concentrations from about 1 to 25 μM. Preferred is a range of about 2 to about 7 μM. PMA and related molecules, used to increase proliferation, may be used at a concentration of about 1 μg/ml to 500 μg/ml, preferably at a concentration of about 10 μg/ml to 200 μg/ml. The glycosaminoglycan, heparan sulfate, is a ubiquitous component on the surface of mammalian cells known to affect a variety of cellular processes, and which binds to growth factor molecules such as FGF and amphiregulin, thereby promoting the binding of these molecules to their receptors on the surfaces of cells. It can be added to the culture medium in combination with other biological factors, at a concentration of about 1 ng/ml to 1 mg/ml; more preferred is a concentration of about 0.2 μg/ml to 20 μg/ml, most preferred is a concentration of about 2 μg/ml.

Using these screening methods, it is possible to screen for potential drug side-effects on pre- and post-natal CNS cells by testing for the effects of the biological agents on stem cell and progenitor cell proliferation and on progenitor cell differentiation or the survival and function of differentiated CNS cells. The proliferated precursor cells are typically plated at a density of about $5–10\times10^6$ cells/ml. If it is desired to test the effect of the biological agent on a particular differentiated cell type or a given make-up of cells, the ratio of neurons to glial cells obtained after differentiation can be manipulated by separating the different types of cells. For example, the O4 antibody (available from Boerhinger Mannheim) binds to oligodendrocytes and their precursors. Using a panning procedure, oligodendrocytes are separated out. Astrocytes can be panned out after a binding procedure using the RAN 2 antibody (available from ATCC). Tetanus toxin (available from Boerhinger Mannheim) can be used to select out neurons. By varying the trophic factors added to the culture medium used during differentiation it is possible to intentionally alter the phenotype ratios. Such trophic factors include EGF, FGF, BDNF, CNTF, TGFα, GDNF, and the like. For example, FGF increases the ratio of neurons, and CNTF increases the ratio of oligodendrocytes. Growing the cultures on beds of glial cells obtained from different CNS regions will also affect the course of differentiation as described above. The differentiated cultures remain viable (with phenotype intact) for at least a month.

The effects of the biological agents are identified on the basis of significant difference relative to control cultures with respect to criteria such as the ratios of expressed phenotypes (neurons: glial cells, or neurotransmitters or other markers), cell viability and alterations in gene expression. Physical characteristics of the cells can be analyzed by observing cell and neurite morphology and growth with microscopy. The induction of expression of new or increased levels of proteins such as enzymes, receptors and other cell surface molecules, or of neurotransmitters, amino acids, neuropeptides and biogenic amines can be analyzed with any technique known in the art which can identify the alteration of the level of such molecules. These techniques include immunohistochemistry using antibodies against such molecules, or biochemical analysis. Such biochemical analysis includes protein assays, enzymatic assays, receptor binding assays, enzyme-linked immunosorbant assays (ELISA), electrophoretic analysis, analysis with high performance liquid chromatography (HPLC), Western blots, and radioimmune assays (RIA). Nucleic acid analysis such as Northern blots and PCR can be used to examine the levels of mRNA coding for these molecules, or for enzymes which synthesize these molecules.

The factors involved in the proliferation of stem cells and the proliferation, differentiation and survival of stem cell progeny, and/or their responses to biological agents can be isolated by constructing cDNA libraries from stem cells or stem cell progeny at different stages of their development using techniques known in the art. The libraries from cells at one developmental stage are compared with those of cells at different stages of development to determine the sequence of gene expression during development and to reveal the effects of various biological agents or to reveal new biological agents that alter gene expression in CNS cells. When the libraries are prepared from dysfunctional tissue, genetic factors may be identified that play a role in the cause of dysfunction by comparing the libraries from the dysfunctional tissue with those from normal tissue. This information can be used in the design of therapies to treat the disorders. Additionally, probes can be identified for use in the diagnosis of various genetic disorders or for use in identifying neural cells at a particular stage in development.

Electrophysiological analysis can be used to determine the effects of biological agents on neuronal characteristics such as resting membrane potential, evoked potentials, direction and ionic nature of current flow and the dynamics of ion channels. These measurements can be made using any technique known in the art, including extracellular single unit voltage recording, intracellular voltage recording, voltage clamping and patch clamping. Voltage sensitive dyes and ion sensitive electrodes may also be used.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the scope of the invention, as defined by the appended claims.

EXAMPLE 1: Dissociation of Embryonic Neural Tissue

14-day-old CD_1 albino mouse embryos (Charles River) were decapitated and the brain and striata were removed using sterile procedure. Tissue was mechanically dissociated with a fire-polished Pasteur pipette into serum-free medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient (Gibco). Dissociated cells were centrifuged at 800 r.p.m. for 5 minutes, the supernatant aspirated, and the cells resuspended in DMEM/F-12 medium for counting.

EXAMPLE 2: Dissociation of Adult Neural Tissue

Brain tissue from juvenile and adult mouse brain tissue was removed and dissected into 500 µm sections and immediately transferred into low calcium oxygenated artificial cerebrospinal fluid (low Ca^{2}+ aCSF) containing 1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid. Tissue was stirred in this solution for 90 minutes at 32° C.-35° C. aCSF was poured off and replaced with fresh oxygenated aCSF for 5 minutes. Tissue was transferred to DMEM/F-12/10% hormone solution containing 0.7 mg/ml ovomucoid and triturated with a fire polished pasteur pipette. Cells were centrifuged at 400 rpm. for 5 minutes, the supernatant aspirated and the pelleted cells resuspended in DMEM/F-12/10% hormone mix.

EXAMPLE 3: Proliferation of Neural Stem Cells on Substrates

2500 cells/cm^2 prepared as in Example 1 were plated on poly-L-ornithine-coated (15 µg/ml;Siqma) glass coverslips in 24 well Nunclon (0.5 ml/well) culture dishes. The culture medium was a serum-free medium composed of DMEM/F-12 (1:1) including glucose (0.6%), glutamine (2 gM), sodium bicarbonate (3 mM), and HEPES (4-[2hydroxyethyl]-1-piperazineethanesulfonic acid) buffer (5 mM) (all from Sigma except glutamine [Gibco]). A defined hormone mix and salt mixture (Sigma) that included insulin (25 µg/ml), transferrin (100 µg/ml), progesterone (20 nM), putrescine (60 µM), and selenium chloride (30 nM) was used in place of serum. Cultures contained the above medium, hereinafter referred to as "Complete Medium" together with 16-20 ng/ml EGF (purified from mouse sub-maxillary, Collaborative Research) or TGFα (human recombinant, Gibco). After 10-14 days in vitro, media (DMEM only plus hormone mixture) and growth factors were replaced. This medium change was repeated every two to four days. The number of surviving cells at 5 days in vitro was determined by incubating the coverslips in 0.4% trypan blue (Gibco) for two minutes, washing with phosphate buffered saline (PBS, pH 7.3) and counting the number of cells that excluded dye with a Nikon Diaphot inverted microscope.

EXAMPLE 4: Proliferation of Embryonic Mouse Neural Stem Cells in Suspension

Dissociated mouse brain cells prepared as in Examples 1 and 2 (at 1×10^5 cell/ml) were suspended in Complete Medium with 20 ng/ml of EGF or TGFα. Cells were seeded in a T25 culture flask and housed in an incubator at 37° C., 100% humidity, 95% air/5% CO_2. Cells began to proliferate within 3-4 days and due to a lack of substrate lifted off the floor of the flask and continued to proliferate in suspension forming clusters of undifferentiated cells, referred to herein as "neurospheres". After 6-7 days in vitro the proliferating clusters (neurospheres) were fed every 2-4 days by gentle centrifugation and resuspension in DMEM with the additives described above.

EXAMPLE 5: Proliferation of Adult Mouse Neural Stem Cells in Suspension

The striata, including the subependymal region, of female, pathogen-free CD1 albino mice [3 to 18 month old; Charles River (CF1 and CF2 strains yielded identical results)] were dissected and hand cut with scissors into 1-mm coronal sections and transferred into aCSF (pH 7.35, approx. 180 mOsmol), aerated with 95% O_2-5% CO_2 at room temperature. After 15 minutes the tissue sections were transferred to a spinner flask (Bellco Glass) with a magnetic stirrer filled with low-Ca^{2}+ aCSF (pH 7.35, approx. 180 mOsmol), aerated with 95% O_2-5% CO_2 at 32° to 35° C., containing 1.33 mg/ml of trypsin (9000 BAEE units/mg), 0.67 mg/ml of hyaluronidase (2000 units/mg) and 0.2 mg/ml of kynurenic acid. After 90 minutes, tissue sections were transferred to normal aCSF for 5 minutes prior to trituration. Tissue was transferred to DMEM/F-12 (A: 1, Gibco) medium containing 0.7 mg/ml ovomucoid (Sigma) and triturated mechanically with a fire-narrowed pasteur pipet. Cells were plated (1000 viable cells per plate) in noncoated 35 mm culture dishes (Costar) containing Complete Medium and EGF [20 ng/ml, purified from mouse sub-maxillary gland (Collaborative Research)] or human recombinant (Gibco/BRL). Cells were allowed to settle for 3-10 minutes after which the medium was aspirated away and fresh DMEM/F-12/hormone mix/EGF was added. After 5-10 days in vitro the number of spheres (neurospheres) were counted in each 35 mm dish.

EXAMPLE 6: Passaging Proliferated Stem Cells

After 6-7 days in vitro, individual cells in the neurospheres from Example 4 were separated by triturating the neurospheres with a fire polished pasteur pipette. Single cells from the dissociated neurospheres were suspended in tissue culture flasks in DMEM/F-12/10% hormone mix together with 20 ng/ml of EGF. A percentage of dissociated cells began to proliferate and formed new neurospheres largely composed of undifferentiated cells. The flasks were shaken well and neurospheres were allowed to settle in the bottom corner of the flask. The neurospheres were then transferred to 50 ml centrifuge tubes and centrifuged at 300 rpm for 5 minutes. The medium was aspirated off, and the neurospheres were resuspended in 1 ml of medium containing EGF. The cells were dissociated with a fire-narrowed pasteur pipette and triturated forty times. 20 microliters of cells were removed for counting and added to 20 microliters of Trypan Blue diluted 1:2. The cells were counted and replated at 50,000 cells/ml. This procedure can be repeated weekly and results in a logarithmic increase in the number of viable cells at each passage. The procedure is continued until the desired number of stem cell progeny is obtained.

EXAMPLE 7. Differentiation of Neural Stem Cell Progeny and Immunocytochemistry

Cells proliferated from Examples 4 and 6 were induced to differentiate by maintaining the cells in the culture flasks in the presence of EGF or TGFα at 20 ng/ml without reinitiating proliferation by dissociation of the neurospheres or by plating on poly-ornithine in the continued presence of EGF or TGFα.

Indirect immunocytochemistry was carried out with cells prepared as in Example 3 which had been cultured for 14-30 days in vitro on glass coverslips. For anti-NSE (or anti-nestin) and anti-GFAP immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS and 95% ethanol/5% acetic acid, respectively. Following a 30 minute fixation period, coverslips were washed three times (10 minutes each) in PBS (pH=7.3) and then incubated in the primary antiserum (NSE 1:300, nestin 1:1500 or GFAP 1:100) in PBS/10% normal goat serum/0.3% TRITON®-X-100) for two hours at 37° C. Coverslips were washed three times (10 minutes each) in PBS and incubated with secondary antibodies (goat-anti-rabbit-rhodamine for anti-NSE or anti-nestin and goat-anti-mouse-fluorescein for antiGFAP, both at 1:50) for 30 minutes at 37° C. Coverslips were then

washed three times (10 minutes each) in PBS, rinsed with water, placed on glass slides and coverslipped using Fluorsave, a mounting medium preferable for use with fluorescein-conjugated antibodies. Fluorescence was detected and photographed with a Nikon Optiphot photomicroscope.

Neural stem cell progeny were also differentiated using the following differentiation paradigms. The neurospheres used for each paradigm were generated as outlined in Examples 4 and 6. All the neurospheres used were passaged at least once prior to their differentiation.

Paradigm 1—Rapid differentiation of neurospheres

Six to 8 days after the first passage, the neurospheres were removed and centrifuged at 400 r.p.m. The EGF-containing supernatant was removed and the pellet suspended in EGF-free complete medium containing 1% FBS. Neurospheres (approximately $0.5–1.0\times10^6$ cells/well) were plated on poly-L-ornithine-coated (15 μg/ml) glass coverslips in 24 well Nuclon (1.0 ml/well) culture dishes. After 24 hours in culture, the coverslips were transferred to 12 well (Costar) culture dishes containing complete medium containing 0.5% FBS. The medium was changed every 4–7 days. This differentiation procedure is referred to as the "Rapid Differentiation Paradigm" or RDP.

Paradigm 2—Differentiation of dissociated neurospheres

Six to 8 days after the first passage, the neurospheres were removed and centrifuged at 400 r.p.m. The EGF-containing media was removed and the pellet was suspended in EGF-free complete medium containing 1% FBS. The neurospheres were mechanically dissociated into single cells with a fire-polished Pasteur pipette and centrifuged at 800 r.p.m. for 5 minutes. Between 0.5×10^6 and 1.0×10^6 cells were plated on poly-L-ornithine-coated (15 μg/ml) glass coverslips in 24 well Nuclon (1.0 ml/well) culture dishes. The EGF-free culture medium containing 1% FBS was changed every 4–7 days.

Paradigm 3—Differentiation of single neurospheres

Neurospheres were washed free of EGF by serial transfers through changes of EGF-free medium. A single neurosphere was plated onto poly-L-ornithine-coated (15 μg/ml) glass coverslips in a 24-well plate. The culture medium used was complete medium with or without 1% FBS. The medium was changed every 4–7 days.

Paradigm 4—Differentiation of single dissociated neurospheres

Neurospheres were washed free of EGF by serial transfers through changes of EGF-free medium. A single neurosphere was mechanically dissociated in a 0.5 ml Eppendorf centrifuge tube and all the cells were plated onto a 35 mm culture dish. Complete medium was used with or without 1% FBS.

Paradigm 5—Differentiation of neurospheres co-cultured with striatal astrocytes

Neurospheres, derived from striatal cells as described in Example 1 were labeled with 5-bromodeoxyuridine (BrdU) and washed free of EGF. An astrocyte feeder layer was generated from striatal tissue of postnatal mice (0–24 hours), and plated on poly-L-ornithine-coated glass coverslips in a 24-well culture dish. When the astrocytes were confluent, a dissociated or intact neurosphere was placed on each astrocyte bed. Complete medium was changed after the first 24 hours and then every forty-eight hours. When differentiated on an astrocyte feeder layer, in addition to GABAergic and substance P-ergic neurons, somatostatin, NPY, glutamate and methenkephalin-containing neurons were present.

EXAMPLE 8: Effect of Growth Factors on Neurosphere Differentiation

The effects of CNTF, FGF-2, BDNF, and retinoic acid on neurosphere differentiation were tested using the differentiation paradigms set forth in Example 7.

CNTF

The effect of CNTF was assayed in paradigms 1 and 3. For both paradigms, CNTF was added either at the beginning of the experiment at a concentration of 10 ng/ml or daily at a concentration of 1 ng/ml. In paradigm 1, the addition of CNTF increased the number of NSE-immunoreactive cells in addition to the number of tau-1-immunoreactive cells, suggesting that CNTF has an effect on the proliferation, survival, or differentiation of neurons. Preliminary testing with antibodies recognizing the neurotransmitters GABA and substance P suggest that there is no increase in the number of cells containing these proteins. This suggests that a different neuronal phenotype is being produced.

Three different antibodies directed against O4, galactocerebroside (GalC) and MBP were used to study the effect of CNTF on the oligodendrocytes of paradigm 1. CNTF had no effect on the number of O4(+) cells, but there was an increase in the number of GalC(+) and MBP(+) cells compared with the control. Thus it appears that CNTF plays a role in the maturation of oligodendrocytes.

In one experiment, the neurospheres were differentiated as outlined in paradigm 1 except that serum was never added to the culture medium. While the effect of CNTF on neurons and oligodendrocytes was not as apparent as in the presence of serum, there was an increase in the proliferation of flat, protoplasmic astrocytes. Hence, CNTF will affect astrocyte differentiation in various culture conditions.

In paradigm 3, the addition of CNTF resulted in an increase in the number of NSE(+) cells.

BDNF

The effect of BDNF was tested using Paradigm 3. There was an increase in the number of NSE(+) neurons per neurosphere. Additionally, there was an increase in the neuronal branching and the migration of the neurons away from the sphere.

FGF-2

The effect of FGF-2 was tested using paradigms 2 and 4. In paradigm 2, 20 ng/ml of FGF-2 was added at the beginning of the experiment and cells were stained 7 days later. FGF-2 increased the number of GFAP(+) cells and the number of NSE(+) cells. This suggests that FGF-2 has a proliferative or survival effect on the neurons and astrocytes.

In paradigm 4, 20 ng/ml of FGF-2 was added at the beginning of the experiment and assayed 7–10 days later. FGF-2 induced the proliferation of neural stem cell progeny generated by the EGF-responsive stem cell. It induced two different cell types to divide, neuroblasts and bipotential progenitor cells. The neuroblast produced, on average, 6 neurons while the bipotential cell produced approximately 6 neurons and a number of astrocytes.

In previous studies, it was found that when plated at low density (2500 cells/cm^2), addition of EGF up to 7 days in vitro (DIV) could initiate proliferation of the stem cell, but not if applied after 7 DIV. Striatal cells (E14, 2500 cell/cm^2) were plated in the absence or presence of 20 ng/ml of FGF-2. After 11 DIV, cultures were washed and medium containing 20 ng/ml of EGF was added. After 4–5 DIV, in cultures that were primed with FGF-2, greater than 70% of the wells examined contained clusters of proliferating cells that, developed into colonies with the morphologic and antigenic properties of the EGF-generated cells. Cultures that had not been primed with FGF-2 showed no EGF-responsive proliferation. These findings suggest that the EGF-responsive stem cells possess FGF-2 receptors that regulate its long term survival.

Retinoic acid

The effect of retinoic acid at 10^{-7}M was tested using paradigm 1. There was an increase in the number of NSE(+) and tau-1(+) cells, suggesting that retinoic acid increases the number of neurons.

EXAMPLE 9: Proliferation of Embryonic Human Neural Stem Cells and Differentiation of the Neural Stem Cell Progeny

With approval of the Research Ethical Committee at the University of Lund and the Ethics Committee at the University of Calgary, nine 8–12 week old human fetuses were obtained by suction abortions. Tissue was dissected and any identifiable brain regions were removed. Within 4–5 days post-dissection, tissue pieces were mechanically dissociated into single cells using the procedure of Example 1 and the number of viable cells was counted. About 0.1×10^6–0.5×10^6 cells were plated in 35 cm^2 tissue culture flasks (without substrate pretreatment) in Complete Medium with 20 ng/ml of human recombinant EGF (Gibco/BRL).

Two to three days after plating the cells, the majority of the viable cells had extended processes and had taken on a neuronal morphology. By seven days in vitro (DIV), the neuronal-like cells began to die and by 14 DIV nearly all of these cells were dead or dying (determined by the absence of processes, irregular membranes and granular cytoplasm). A few of the cells (1%) did not extend any processes or flatten nor did they take on an astrocytic morphology, instead these cells remained rounded and by 5 to 7 DIV began to divide. By 10 to 14 DIV, small clusters of cells, attached to the substrate, were identified. During the next 7 to 10 days (17 to 24 DIV), these small clusters continued to grow in size and many remained attached to the substrate. By 28 to 30 DIV, nearly all the proliferating clusters had lifted off the substrate and were floating in suspension. While floating in suspension, the clusters continued to grow in size and were passaged after they had been in culture for 30 to 40 days using the procedure described in Example 6. EGF-responsive cells began to proliferate after a few DIV and formed floating spheres that were passaged a second time after 30 to 40 DIV.

Thirty to 60 days after passage two or three, 2–3 ml aliquots containing media and pass 2 spheres were taken from the tissue culture flasks and plated onto 35 mm culture dish. Single spheres were placed onto poly-L-ornithine coated glass coverslips in DMEM/F-12/HM medium containing EGF. Spheres immediately attached to the substrate and within the first 24–48 hours cells begin to migrate from the sphere. At 14 DIV cells continued to proliferate and migrate resulting in an increase in the diameter of the transferred sphere. By 30 DIV, a large number of cells had been generated from the original sphere and had migrated at a similar rate from the center producing a concentric circle of associated cells. At the periphery, the majority of the cells were one cell layer thick while closer to the center there were denser regions of cells.

Forebrain regions from eight week old tissue produced no spheres, while spheres were observed from hindbrain tissue in two of the four eight week old samples. For the nine week old fetuses, spheres were generated from forebrain region in two of the four samples and in two of the three hindbrain regions which were received. The twelve week old fetus contained only hindbrain tissue and spheres were produced.

Spheres generated from primary culture or pass 1 spheres were removed from the tissue culture flask, without inducing differentiation, and plated onto poly-L-ornithine coated glass coverslips in DMEM/F-12/HM medium for two hours to allow the spheres to attach to the substrate. Coverslips were removed and processed for indirect immunohistochemistry. Immunostaining with antibodies directed against neurofilaments (168 kDa) or GFAP did not identify any immunoreactive (IR) cells. However, nearly all of the cells were immunoreactive with an antibody that recognizes human nestin.

Thirty to 45 days after being plated onto the poly-L-ornithine coated substrate, cells were fixed and processed for indirect immunocytochemical analysis with antibodies directed against: MAP-2, Tau-1, neurofilament 168 kDA, GABA, substance P (neuronal markers); GFAP (astrocyte marker); O4 and MBP (oligodendrocyte markers). Numerous MAP-2 and Tau-1-IR cell bodies and processes were identified in addition to a large number of Tau-1-IR fibers. While there was no indication of substance P immunoreactivity, GABA-IR cell bodies with long branched processes were seen. Neurofilament-IR cells were strongly IR for GFAP. O4-IR cells with an O-2A morphology and an oligodendrocyte morphology were present. MBP-IR (found on oligodendrocytes) was also seen throughout the cultures.

EXAMPLE 10: Proliferation of Adult Monkey (Rhesus) Neural Stem Cells and Differentiation of the Neural Stem Cell Progeny

The conus medularis was removed from an adult male monkey (Rhesus) and hand cut with scissors into 1-mm sections and transferred into artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (pH 7.35, approx. 280 mOsmol), aerated with 95% O_2–5% CO_2 at room temperature. After 15 min, the tissue sections were transferred to a spinner flask (Bellco Glass) with a magnetic stirrer filled with low-Ca^{2+} aCSF containing 124 mM NaCl, 5 mM KCl, 3.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (pH 7.35, approx. 280 mOsmol), aerated with 95% O_2–5% CO_2 at 32° to 35° C., containing 1.33 mg/ml of trypsin (9000 BAEE units/mg), 0.67 mg/ml of hyaluronidase (2000 units/mg) and 0.2 mg/ml of kynurenic acid. After 90 min, tissue sections were transferred to normal aCSF for 5 min prior to trituration. Tissue was transferred to DMEM/F-12 (1:1, Gibco) medium containing 0.7 mg/ml ovomucoid (Sigma) and triturated mechanically with a fire-narrowed pasteur pipet.

Cells were plated (1000 viable cells per plate) in non-coated 35 mm culture dishes (Costar) containing Complete Medium and 20 ng/ml EGF (human recombinant from Gibco/BRL). After 7 to 10 days in culture, floating spheres

were transferred with wide-bore pipets onto laminin (15 μg/ml)(Sigma)-coated glass coverslips in 24-well culture dishes. EGF@20 ng/ml was added to the medium. Spheres attached to the substrate and cells within the sphere continued to proliferate. After 14 to 21 days in vitro (DIV), the cells were probed by indirect immunocytochemistry for the presence of neuron, astrocytes and oligodendrocytes. All three cell types were identified.

EXAMPLE 11: Proliferation of Adult Human Neural Stem Cells and Differentiation of the Neural Stem Cell Progeny

During a routine biopsy, normal tissue was obtained from a 65 year old female patient. The biopsy site was the right frontal lobe, 6 mm from the tip of the frontal/anterior horn of the lateral ventricle. The tissue was dissociated using the procedure outlined in Example 2 and cultured in Complete Medium with EGF and FGF-2 (20 ng/ml of each growth hormone), in T25 flasks (Nunclon). The flasks were examined every 2–3 days for neurosphere formation. Clonally-derived cells were passaged using single sphere dissociation: single neurospheres were triturated 100× in sterile aliquot tubes containing 200 μl of the media/hormone/EGF-FGF-2 solution before culturing in 24- or 96-well plates. First-passage neurospheres were plated on poly-ornithine and laminin coated coverslips and allowed to plate down for 14 days in media/hormone/EGF+FGF-2. Some first passage neurospheres were plated on laminin (20 μg/ml) and poly-ornithine coated coverslips in media/hormone mix for 19 hours, then processed for nestin staining as outlined in Example 7. Nestin staining indicated that the neurospheres, prior to the induction of differentiation (as described below) were nestin positive, indicative of the presence of immature undifferentiated cells.

Pass one human neurospheres were plated on a laminin coated substrate (see above). After 14 days, the cultures received a media change to media/hormone mix plus 1% FBS and were allowed to differentiate for 7 days. Immunocytochemical analysis was then performed to determine different neural phenotypes. The differentiated cells were fixed with 4% paraformaldehyde in PBS for 20 minutes. The coverslips were washed three times (five minutes each) in PBS. For triple label immunocytochemistry, the cells were permeabilized for 5 minutes in 0.3% TRITON®-X in PBS followed by 2 washes with PBS. A first set of primary antibodies, MAP-2 (mouse monoclonal, 1:1000, Boerhinger Mannheim) and GFAP (Rabbit polyclonal, 1:300, BTI), used to determine the presence of neurons and astrocytes respectively, were mixed in 10% normal goat serum in PBS. The cells were incubated at 37° C. for 2 hours and then washed 3 times in PBS. A first set of secondary antibodies, goat anti-mouse rhodamine (Jackson Immuno Research) and goat anti-rabbit FITC (IgG, 1:100 Jackson Immuno Research) were mixed in PBS. The cells were incubated for 30 minutes at 37° C. and then rinsed three times with PBS. The second primary antibody, O4 (mouse monoclonal IgM, 1:100) for oligodendrocytes, was mixed in 10% normal goat serum in PBS. The cells were incubated for 2 hours at 37° C. The second set of secondary goat anti-mouse AMCA IgM (1:100 Jackson Immuno Research) was mixed in PBS and cells were incubated for 30 minutes at 37° C. The cells were then rinsed twice in PBS and then in double distilled water before mounting with Fluorosave.

EXAMPLE 12: Screening for the trkB Receptor on Neural Stem Cell Progeny

The expression of the trk family of neurotrophin receptors in EGF-generated neurospheres was examined by northern blot analysis. Total mRNA was isolated from mouse and rat striatal sGF-generated neurospheres. Both rat and mouse neurospheres expressed high levels of trkB receptor mRNA, but did not express trk nor trkc mRNA. In preliminary experiments, single EGF-generated mouse neurospheres were plated on poly-L-ornithine coated glass coversips and cultured in the absence or presence of 10ng/ml of BDNF. When examined after 14–28 days in vitro, neurospheres plated in the presence of BDNF contained NSE(+) cells with extensive and highly branched processes; well-developed NSE(+) cells were not observed in the absence of BDNF. Activation of the trkB receptor on EGF-generated neurospheres may enhance differentiation, survival of and/or neurite outgrowth from newly generated neurons.

EXAMPLE 13: Screening for the GAP-43 Membrane Phosphoprotein on Neural Stem Cell Progeny

Growth-associated protein (GAP-43) is a nervous system-specific membrane phosphoprotein which is down-regulated during development. Originally, GAP-43 was though to be neuron-specific, however, recent reports indicate that this protein may be at least transiently expressed during development in some astrocytes, oligodendrocytes and in Schwann cells. At present, the role of GAP-43 in macroglia is not known. The transient expression of GAP-43 in glial cells generated from the EGF-responsive stem cells derived from embryonic and adult murine striatum was investigated. Glial cell (astrocyte and oligodendrocyte) differentiation was induced by plating neural stem cell progeny in a medium containing 1% FBS with no EGF. The cells were then probed with specific antibodies for GAP-43, nestin, GFAP, O4, and GalC. In order to identify cells expressing GAP-43, the antibodies were pooled in various combinations using dual-label immunofluorescence methods.

During the first two days post plating, there was a low to moderate level of GAP43 expression in almost all cells (flat, bipolar and stellate), but by 3–4 days post-plating, the level of GAP-43 expression became restricted to the bipolar and stellate cells. At 4 days the majority of GAP-43-expressing cells co-labelled with the oligodendrocyte markers O4 and GalC although GFAP and GAP-43 was coexpressed in a number of cells. At one week post-plating however, essentially all of the GFAP-expressing astrocytes no longer expressed GAP-43 while the majority of the O4 and GalC-expressing cells continued to express GAP-43. At 7–10 days, these oligodendrocytes began to express MBP and lose the expression of GAP-43. The EGF-responsive stem cells may represent a useful model system for the study of the role of GAP-43 in glial and neuronal development.

EXAMPLE 14: Treatment of Neurodegenerative Disease Using Progeny of Human Neural Stem Cells Proliferated In Vitro

Cells are obtained from ventral mesencephalic tissue from a human fetus aged 8 weeks following routine suction abortion which is collected into a sterile collection apparatus. A 2×4×1 mm piece of tissue is dissected and dissociated as in Example 1. Neural stem cells are then proliferated as in Example 4. Neural stem cell progeny are used for neurotransplantation into a blood-group matched host with a neurodegenerative disease. Surgery is performed using a BRW computed tomographic (CT) stereotaxic guide. The patient is given local anesthesia suppiemencea with intravenously administered midazolam. The patient undergoes CT scanning to establish the coordinates of the region to

receive the transplant. The injection cannula consists of a 17-gauge stainless steel outer cannula with a 19-gauge inner stylet. This is inserted into the brain to the correct coordinates, then removed and replaced with a 19-gauge infusion cannula that has been preloaded with 30 μl of tissue suspension. The cells are slowly infused at a rate of 3 μl/min as the cannula is withdrawn. Multiple stereotactic needle passes are made throughout the area of interest, approximately 4 mm apart. The patient is examined by CT scan postoperatively for hemorrhage or edema. Neurological evaluations are performed at various post-operative intervals, as well as PET scans to determine metabolic activity of the implanted cells.

EXAMPLE 15: Remyelination of Myelin Deficient Rats Using Neural Stem Cell Progeny Proliferated In Vitro

Embryonic day 15 (E15) Sprague Dawley rats and E14–15 mice were obtained and the neural tissue was prepared using the methods described in Example 1. The cells were suspended in Complete Medium with 16–20 ng/ml EGF (purified from mouse submaxillary, Collaborative Research) or TGFα (human recombinant, Gibco). The cells were seeded in a T25 culture flask and housed in an incubator at 37° C., 100% humidity, 95% air/5% CO_2 and proliferated using the suspension culture method of Example 4. Cells proliferated within 3–4 days and, due to lack of substrate, lifted off the floor of the flask and continued to proliferate in suspension forming neurospheres.

After 6–8 days in vitro (DIV) the neurospheres were removed, centrifuged at 400 r.p.m. for 2–5 minutes, and the pellet mechanically dissociated into individual cells with a fire-polished glass pasteur pipet. Cells were replated in the growth medium where proliferation of the stem cells and formation of new neurospheres was reinitiated.

Litters of first day postnatal myelin deficient rats were anesthetized using ice to produce hypothermia. Myelin deficiency is an X-linked trait and thus only one half of the males in any litter are affected. Therefore, only the males were used for these studies. Once anesthetized, a small rostral to caudal incision was made at the level of the lumbar enlargement. The muscle and connective tissue was removed to expose the vertebral laminae. Using a fine rat tooth forceps, one lamina at the lumbar enlargement was removed and a small cut is made in the dura mater to expose the spinal cord.

A stereotaxic device holding a glass pipet was used to inject a 1 μl aliquot of the cell suspension (approximately 50,000 cells/μl) described above. The suspension is slowly injected into a single site (although more could be done) in the dorsal columns of the spinal cord. As controls, some of the animals were sham-injected with sterile saline. The animals were marked by clipping either toes or ears to distinguish between both experimental groups. Following injection of the cell suspension, the wound was closed using sutures or stainless steel wound clips and the animals were revived by warming on a surgical heating pad and then returned to their mother.

The animals were allowed to survive for two weeks post-injection and were then deeply anesthetized with nembutal (150 mg/kg) and perfused through the left ventricle. The spinal cords were removed and the tissue examined by light and electron microscopy. Patches of myelin were found in the dorsal columns of the recipients of both rat and mouse cells, indicating that neural stem cells isolated from rat and mouse neural tissue can differentiate into oligodendroglia and are capable of myelination in vivo.

Because the myelin deficient rat spinal cord is almost completely devoid of myelin, myelin formed at or near the site of injection is derived from the implanted cells. It is possible that the process of injection will allow for the entry of Schwann cells (myelinating cells of the PNS) into the spinal cord. These cells are capable of forming myelin within the CNS but can be easily distinguished from oligodendrocytes using either light microscopy or immunocytochemistry for CNS myelin elements. There is usually a very small amount of CNS myelin within the myelin deficient rat spinal cord. This myelin can be distinguished from normal donor myelin based on the mutation within the gene for the major CNS myelin protein, proteolipid protein (PLP). The myelin deficient rat myelin is not immunoreactive for PLP while the donor myelin is.

EXAMPLE 16: Remyelination in Human Neuromyelitis Optica

Neuromyelitis optica is a condition involving demyelination of principally the spinal cord and optic nerve. Onset is usually acute and in 50% of the cases death occurs within months. The severity of demyelination as well as lesion sites can be confirmed by magnetic resonance imaging (MRI).

Neural stem cell progeny are prepared from fetal human tissue by the methods of Example 9 or 14. Cells are stereotactically injected into the white matter of the spinal cord in the vicinity of plaques as visualized by MRI. Cells are also injected around the optic nerve as necessary. Booster injections may be performed as required.

EXAMPLE 17: Remyelination in Human Pelizaeus-Merzbacher Disease

Pelizaeus-Merzbacher disease is a condition involving demyelination of the CNS. The severity of demyelination as well as lesion sites can be confirmed by magnetic resonance imaging (MRI).

Neural stem cell progeny are prepared from fetal human tissue by the methods of Examples 9 or 14. Cells are stereotactically injected into the white matter of the spinal cord in the vicinity of plaques as visualized by MRI. Cells are also injected around the optic nerve as necessary. Booster injections may be performed as required.

EXAMPLE 18: Genetic Modification of Neural Stem Cell Progeny

Cells proliferated as in Examples 3 or 4 are transfected with expression vectors containing the genes for the FGF-2 receptor or the NGF receptor. Vector DNA containing the genes are diluted in 0.1X TE (1 mM Tris pH 8.0, 0.1 mM EDTA) to a concentration of 40 μg/ml. 22 μl of the DNA is added to 250 μl of 2X HBS (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4.2H_2O$, 12 mM dextrose, 50 mM HEPES) in a disposable, sterile 5 ml plastic tube. 31 μl of 2M $CaCl_2$ is added slowly and the mixture is incubated for 30 minutes at room temperature. During this 30 minute incubation, the cells are centrifuged at 800 g for 5 minutes at 4° C. The cells are resuspended in 20 volumes of ice-cold PBS and divided into aliquots of 1×10^7 cells, which are again centrifuged. Each aliquot of cells is resuspended in 1 ml of the DNA-$CaCl_2$ suspension, and incubated for 20 minutes at room temperature. The cells are then diluted in growth medium and incubated for 6–24 hours at 37° C. in 5%–7% CO^2. The cells are again centrifuged, washed in PBS and returned to 10 ml of growth medium for 48 hours.

The transfected neural stem cell progeny are transplanted into a human patient using the procedure described in

Example 14, or are used for drug screening procedures as described in the examples below.

EXAMPLE 19: Genetic Modification of Neural Stem Cell Progeny With a Retrovirus Containing the Bacterial B-Galactosidase Gene

Neural stem cell progeny were propagated as described in Example 4. A large pass-1 flask of neurospheres (4–5 days old) was shaken to dislodge the spheres from the flask. The flask was spun at 400 r.p.m. for 3–5 minutes. About half of the media was removed without disturbing the neurospheres. The spheres and the remaining media were removed, placed into a Falcon 12 ml centrifuge tube, and spun at 600 r.p.m. for 3–5 minutes. The remaining medium was removed, leaving a few hundred microliters.

A retrovirus which contained the bacterial B-galactosidase gene was packaged and secreted, in a replication-deficient fashion, by the CRE BAG2 cell line produced by C. Cepko. A day after the CRE cells reached confluence, the cells were washed with PBS and the retrovirus was collected in DMEM/F12/HM/20 ng/ml EGF for four days. The virus-containing media was filtered through a 0.45 μm syringe filter. The neurospheres were resuspended in the virus-containing media, transferred to a large flask, and left in an incubator overnight at 37° C. The next day, the contents of the flask were transferred to a 12 ml centrifuge tube and spun at 800 r.p.m. The cells were resuspended in EGF-containing media/HM, dissociated into single cells, and counted. The cells were replated in a large flask at 50,000 cells/ml in a total of 20 mls.

Four days later, transformed cells were selected with G418 at a concentration of 300 μg/ml. Transformed spheres were plated on a poly-ornithine coated glass coverslip in a 24-well plate. After the neurospheres adhered to the plate, the cells were fixed with 0.1% glutaraldehyde for 5 minutes at 4° C. After the cells were fixed, they were washed twice-with PBS for 10 minutes. The cells were then washed with 0.1% TRITON® in PBS for 10–15 minutes at room temperature. A 1 mg/ml X-Gal solution was added to each well and incubated overnight at 37° C. After incubation overnight, the cells were washed three times with PBS for 10 minutes each and observed for any reaction products. A positive reaction resulted in a blue color, indicating cells containing the transferred gene.

EXAMPLE 20: Proliferation of Neural Stem Cells from Transgenic Mice

Transgenic mice were produced using standard pronuclear injection of the MBP-lacZ chimeric gene, in which the promoter for MBP directs the expression of *E. coli* B-galactosidase (lacZ). Transgenic animals were identified by PCR using oligonucleotides specific for lacZ.

Neurospheres were prepared from E15 transgenic mice and DNA negative littermates using the procedures set forth in Example 4. The neurospheres were propagated in the defined culture medium in the presence of 20 ng/ml EGF and were passaged weekly for 35 weeks. For passaging, the neurospheres were harvested, gently centrifuged at 800 RPM, and mechanically dissociated by trituration with a fire-polished Pasteur pipet. At various passages, the cells were induced to differentiate into oligodendrocytes, astrocytes, and neurons by altering the culture conditions. The free-floating stem cell clusters were gently centrifuged, resuspended in the same base defined medium without EGF and with 1% FBS and plated on poly ornithine-treated glass coverslips to promote cell attachment. The clusters attach firmly to the glass, and the cells slow or stop dividing and begin to differentiate.

The identification of various cell types was accomplished using immunofluorescence microscopy with antibodies specific for neurons (MAP-2, NF-L, and NF-M), astrocytes (GFAP) and oligodendrocytes and oligodendrocyte precursors (A2B5, O_1, O_4, Gal C, and MBP). One to 14 days post-plating, the cells on the coverslips were incubated unfixed, for 30 minutes at room temperature with the primary antibodies O1, O4, GalC, and A2B5 (supernatants) diluted in minimal essential medium with 5% normal goat serum and 25 mM HEPES buffer, pH 7.3 (MEM-HEPES, NGS). Following the primary antibodies, the coverslips were gently washed 5 times in rhodamine-conjugated secondary antibodies (Sigma) diluted in MEM-HEPES, NGS. The coverslips were then washed 5 times in MEM-HEPES and fixed with acid alcohol (5% glacial acetic acid/95% ethanol) at 20° C. The coverslips were then washed 5 times with MEM-HEPES, and either mounted and examined using fluorescence microscopy or immmunoreacted with rabbit polyclonal antisera raised against GFAP, nestin, MBP, or proteolipid protein (PLP). When subjected to a second round of immunolabeling, the coverslips were incubated first for 1 hour with 5% normal goat serum (NGS) in 0.1M phosphate buffer with 0.9% NaCl at pH 7.4 (PBS) and then incubated in rabbit primary antibodies diluted in NGS for 1–2 hours at room temperature. The coverslips were washed 3 times with PBS and then incubated with the appropriate secondary antibody conjugates diluted in NGS, washed again with PBS and then finally mounted on glass microscope slides with Citifluor antifadent mounting medium and examined using a fluorescence microscope. In cases were they were not incubated first with the monoclonal antibody supernatants, the coverslips were fixed for 20 minutes with 4% paraformaldehyde in PBS (pH 7.4), washed with PBS, permeabilized with 100% ethanol, washed again with PBS and incubated with 5% NGS in PBS for 1 hour. The primary antibodies and secondary antibody conjugates were applied as outlined above.

The neural stem cells derived from the transgenic animals were indistinguishable from non transgenic stem cells in their potential for differentiation into neurons, astrocytes, and oligodendrocytes. The MBP promoter directed the expression of the B-galactosidase reporter gene in a cell-specific and developmentally appropriate fashion. The transgene expression is highly stable as oligodendrocytes derived from late passage MBP-lacZ neurospheres (20 passages), expressed the B-galactosidase gene. Thus, transgenically marked neurospheres are likely to be an excellent source of cells for glial cell transplantation.

EXAMPLE 21: Genetic Modification of Neural Stem Cell Progeny Using Calcium Phosphate Transfection

Neural stem cell progeny are propagated as described in Example 4. The cells are then infected using a calcium phosphate transfection technique. For standard calcium phosphate transfection, the cells are mechanically dissociated into a single cell suspension and plated on tissue culture-treated dishes at 50% confluence (50,000–75,000 cells/cm^2) and allowed to attach overnight.

The modified calcium phosphate transfection procedure is performed as follows: DNA (15–25 μg) in sterile TE buffer (10 mM Tris, 0.25 mM EDTA, pH 7.5) diluted to 440 μl with TE, and 60 μl of 2M $CaCl_2$ (pH to 5.8 with 1M HEPES buffer) is added to the DNA/TE buffer. A total of 500 μl of

2×HeBS (HEPES-Buffered saline; 275 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 12 mM dextrose, 40 mM HEPES buffer powder, pH 6.92) is added dropwise to this mix. The mixture is allowed to stand at room temperature for 20 minutes. The cells are washed briefly with 1×HeBS and 1 ml of the calcium phosphate precipitated DNA solution is added to each plate, and the cells are incubated at 37° for 20 minutes. Following this incubation, 10 mls of complete medium is added to the cells, and the plates are placed in an incubator (37° C., 9.5% CO_2) for an additional 3–6 hours. The DNA and the medium are removed by aspiration at the end of the incubation period, and the cells are washed 3 times with complete growth medium and then returned to the incubator.

EXAMPLE 22: Genetically Modified Neural Stem Cell Progeny Expressing NGF

Using either the recombinant retrovirus or direct DNA transfection technique, a chimeric gene composed of the human CMV promoter directing the expression of the rat NGF gene is introduced into the neurosphere cells. In addition, the vector includes the *E. coli* neomycin resistance gene driven off of a viral promoter. After 2 weeks of G418 selection, the cells are cloned using limiting dilution in 96-multi-well plates and the resulting clones are assayed for neurotrophin protein expression using a neurotrophin receptor (trk family) autophosphorylation bioassay.

Clones expressing high levels of NGF are expanded in T-flasks prior to differentiation. The cells are then removed from the EGF-containing complete medium and treated with a combination of serum and a cocktail of growth factors to induce astrocyte differentiation. The astrocytes are again assayed for NGF expression to ensure that the differentiated cells continue to express the trophic factors. Astrocytes that secrete NGF are then injected into fimbria/fornix lesioned rat brains immediately post-lesioning in order to protect the cholinergic neurons. Control astrocytes that do not secrete NGF are injected into similarly lesioned animals. The sparing of cholinergic neurons in the lesion model is assessed using immunocytochemistry for ChAT, the marker for these cholinergic neurons.

EXAMPLE 23: Genetically Modified Neural Stem Cell Progeny Expressing CGAT

Recently, a novel chromaffin granule amine transporter (CGAT) cDNA has been described by Liu et al. (*Cell.* 70:539–551 (1992)), which affords resistance to the neurotoxin MPP+ in Chinese hamster ovary (CHO) cells in vitro. Because dopaminergic neurons from the substantia nigra are specifically killed by MPP+, CGAT gene expression in genetically modified neural stem cell progeny may improve viability of the cells after they are implanted into the Parkinsonian brain. Neural stem cell progeny are propagated as in Example 4. The cells are mechanically dissociated and plated on plastic dishes and infected with a retrovirus containing the CGAT cDNA. The expression of the CGAT cDNA (Liu et al. supra) is directed by a constitutive promoter (CMV or SV40, or a retroviral LTR) or a cell-specific promoter (TH or other dopaminergic or catecholaminergic cell-specific regulatory element or the like). The cells are screened for the expression of the CGAT protein. Selected cells can then be differentiated in vitro using a growth factor or a combination of growth factors to produce dopaminergic or predopaminergic neurons.

EXAMPLE 24: 3H-Thymidine Kill Studies Identify Presence of Constitutively Proliferating Population of Neural Cells in Subependymal Region

Adult male CD1 mice received a series of intraperitoneal injections of 3H-thymidine (0.8 ml per injection, specific activity 45–55 mCi/mmole, ICN Biomedical) on day 0 (3 injections, 1 every 4 hours) in order to kill the constitutively proliferating subependymal cells. On day 0.5, 1, 2, 4, 6, 8 or 12, animals received 2 BrdU injections 1 hour apart (see Example 25) and were sacrificed 0.5 hour after the last injection.

It was observed that 10% of the cells were proliferating on day 1 post-kill, and by 8 days the number of proliferating cells had reached 85%, which was not statistically significantly different from control values. Animals were sacrificed and the brains were removed and processed as described in Example 10.

In a second group of animals, 3H-thymidine injections were given on day 0 (3 injections, 1 every 4 hours), followed by an identical series of injections on day 2 or 4. Animals were allowed to survive for 8 days following the second series of injections (days 9, 10 and 12 respectively) at which time they received 2 injections of BrdU and were sacrificed 0.5 hours later. Animals were sacrificed and the brains were removed and processed as described in Example 25.

After the second series of injections on day 2 only 45% of the proliferating population had returned relative to control values. This indicates that the second series of injections given on day 2 had killed the stem cells as they were recruited to the proliferating mode. The second series of injections given on day 4 resulted in a return to control values by day 8 suggesting that by this time, the stem cells were no longer proliferating and hence were not killed by the day 4 series of injections.

EXAMPLE 25: BrdU Labeling Studies Identifv Presence of Constitutively Proliferating Population of Neural Cells in Subependymal Region

Adult male CD1 mice (25–30 g, Charles River) were injected intraperitoneally (i.p.) with bromodeoxyuridine (BrdU, Sigma, 65 mg/kg) every 2 hours for a total of 5 injections in order to label all of the constitutively proliferating cells in the subependyma lining the lateral ventricles in the forebrain. One month later, animals were sacrificed with an overdose of sodium pentobarbital and transcardially perfused using 4% paraformaldehyde. The brains were removed and post-fixed overnight in 4% paraformaldehyde with 20% sucrose. Brain sections were cut on a cryostat (30 um) and collected in a washing buffer [0.1M phosphate buffered saline (PBS) pH 7.2 with 1% normal horse serum and 0.3% TRITON® X100]. Sections were incubated in 1M HCl at 60° C. for 0.5 hours then washed 3 times (10 minutes each) in washing buffer. Following the final wash, sections were incubated in anti-BrdU (Becton Dickinson, 1:25) for 45 hours at 4° C.. After incubation in the primary antibody, sections were washed 3 times and incubated for 1 hours in biotinylated horse-anti-mouse secondary antibody Dimension Lab, 1:50) at room temperature followed by another 3 washes. The sections were then incubated for 1 hour in avidin conjugated FITC (Dimension Lab, 1:50) at room temperature and washed a final 3 times. Sections were mounted on gelatin coated slides, air-dried and coversnipped with Fluoromount. Slides were examined for BrdU positive cells using a NIKON fluorescent microscope. The number of BrdU positive cells was counted with in the subependyma surrounding the lateral ventricles in 8 samples in sections between the closing of the corpus callosum rostrally and the crossing of the anterior commissure caudally. It was found that 31 days following the series of BrdU injections, 3% of the subependymal cells were still labeled compared to control animals sacrificed immediately following the series of injections (control 100%).

EXAMPLE 26: 3H-Thymidine Kill Studies Identify Presence of Relively Quiescent Neural Stem Cells in Subependymal Region

Adult male CD1 mice were divided into 4 groups. Group A animals received a series of 3H-thymidine injections on day 0 (3 injections, 1 every 4 hours). Animals in groups B and C received a series of 3H-thymidine injections on day 0 followed by a second series of injections on day 2 or 4. Group D animals received injections of physiological saline instead of 3H-thymidine over the same time course a s group A. Animals from all groups were sacrificed by cervical dislocation 16–20 hours following the last series of injections. Brains were removed and neural tissue obtained from the subependyma surrounding the lateral ventricles in the forebrain was dissociated and the neural cells cultured as described in Example 5. At 6 and 8 days in vitro, the total number of spheres was counted in each of the 35 mm wells.

Control animals that received a series of saline injections formed the same number of spheres as animals that received 3H-thymidine on day 0 (which kills the normally proliferating subependymal cells). This indicates that the constitutively proliferating subependymal cells are not the source of stem cells isolated in vitro. Animals that received a second series of injections on day 2 formed 45% the number of spheres (similar to the number of proliferating subependymal cells observed in vivo). When a second series of injections was done on day 4, the number of spheres formed in vitro was not significantly different from control values, again correlating with the in vivo findings. Taken together, this data indicates that the multipotent spheres, which are isolated in vitro in the presence of EGF, are formed from the relatively quiescent stem cell population within the subependyma in vivo.

EXAMPLE 27: In Vivo Proliferation of Neural Stem Cells of Lateral Ventricle

A replication incompetent retrovirus containing the β-galactosidase gene [as described in Walsh and Cepko, *Science* 241:1342, (1988)] was injected into the forebrain lateral ventricles of CD1 adult male mice (25–30 g from Charles River). The injected retrovirus was harvested from the BAG cell line (ATCC CRL-9560) according to the method of Walsh and Cepko (supra). Mice were anesthetized using 65 mg/kg, i.p. sodium pentobarbital. Unilateral stereotactic injections of 0.2–1.0 μl of retrovirus were injected into the lateral ventricle using a 1 μl Hamilton syringe. The coordinates for injection were AP+4.2 mm anterior to lambda, L±0.7 mm, and DV−2.3 mm below dura, with the mouth bar at −2 mm below the interaural line.

On the same day as, one day, or six days following the retrovirus injection, an infusion cannulae attached to a 0.5 μl/hour ALZET osmotic mini-pumps filled with 3.3–330 μg/ml of EGF were surgically implanted into the lateral ventricles at the identical stereotactic coordinates as stated above. The infusion cannula kits were obtained from ALZA. The infusion cannulae were cut to 2.7 mm below the pedestal. The pumps were secured to the mouse skull by use of acrylic cement and a skull screw contralateral and caudal to the injection site. The osmotic mini-pump was situated subcutaneously under and behind the armpit of the left front paw and connected to the infusion cannula by the means of polyethylene tubing.

Six days following initiation of EGF infusion the animals were sacrificed with an overdose of sodium pentobarbital. Mice were transcardially perfused with 2% buffered paraformaldehyde, and the brains were excised and post fixed overnight with 20% sucrose in 2% buffered paraformaldehyde. Coronal slices were prepared with −20 celsius cryostat sectioning at 30 μm. Slices were developed for β-gal histochemistry as per Morshead and Van der Kooy (supra).

Under these conditions, regardless of the day post retrovirus injection, infusion of EGF resulted in an expansion of the population of β-gal labelled cells from an average of 20 cells per brain up to an average of 150 cells per brain and the migration of these cells away from the lining of the lateral ventricles. Infusion of FGF-2 at 33 μg/ml resulted in an increase in the number of β-gal labelled cells, but this increase was not accompanied by any additional migration. Infusion of EGF and FGF together resulted in an even greater expansion of the population of β-gal labelled cells from 20 cells per brain to an average of 350 cells per brain.

These results indicate that FGF may be a survival factor for relatively quiescent stem cells in the subependyma layer, whereas EGF may act as a survival factor for the normally dying progeny of the constitutively proliferating population. The synergistic increase in β-galactosidase cell number when EGF and FGF are infused together further reflects the direct association between the relatively quiescent stem cell and the constitutively proliferating progenitor cell.

EXAMPLE 28: In Vivo Proliferation of Neural Stem Cells of the Third and Fourth Ventricles and the Central Canal

A retroviral construct containing the β-galactosidase gene is microinjected (as in Example 27) into the III ventricle of the diencephalon, IV ventricle of the brain stem and central canal of the spinal cord. Minipumps containing EGF and FGP are then used to continuously administer growth factors for six days (as in Example 27) into the same portion of the ventricular system that the retroviral construct was administered. This produces an increase in the number of β-galactosidase producing cells which survive and migrate out into the tissue near the III ventricle, IV ventricle and central canal of the spinal cord forming new neurons and glia.

EXAMPLE 29: In Vivo Modification and Proliferation of Neural Stem Cells and Differentiation of Neural Stem Cell Progeny of the Lateral Ventricle

A retroviral construct containing the TH gene as well as the β-galactosidase gene is microinjected into the adult lateral ventricle as in Example 27. Mini-pumps containing EGF, FGF, or EGF and FGF together are then used to continuously administer the growth factor(s) into the lateral ventricle for 6 days as in Example 27. As the infected subependymal cells migrate out into the striatum they differentiate into neuronal cells that produce dopamine as measured directly by immunofluorescence with an antibody and (from a direct functional assay) by the ability to overcome the rotational bias produced by unilateral 6-hydroxydopamine lesions.

EXAMPLE 30: In Vivo Infusion of Growth Factors into Ventricles to Obtain Elevated Numbers of Neural Stem Cells

Adult male CD_1 albino mice (30–35 g) from Charles River were anaesthetized with sodium pentobarbital (0.40 mL of a 10% solution) and placed in a stereotaxic apparatus. The dorsal aspect of the skull was exposed with a longitudinal incision. Cannulas were inserted into the fourth ven-

tricle (stereotaxic coordinates A/P −7.0. L±0.3 D/V−5.8), cerebral aqueduct (A/P −4.8 L±D/V−2.6), or central canal (D/V−1.5). The cannulae were attached with sterile tubing to subcutaneous positioned ALZET osmotic mini-pumps containing 25 μg/mL EGF (Becton 40001) and/or 25 μg/mL FGF-2 (R&D Systems 233-FB). Pumps containing sterile saline plus 0.1% mouse albumin (Sigma A3134) were used as controls. The incisions were closed with dental cement. Six days following surgery mice were injected with 0.15 mL BrdU (Sigma B5002); 18 mg/mL in 0.007% NaOH/0.1M PBS) every 2 hours for 8 hours. They were killed 0.5 hours after the last injection with an anaesthetic overdose, and transcardially perfused with 10 mL of ice-cold sterile saline followed by 10 mL of ice-cold Bouin's fixative (5% glacial acetic acid, 9% formaldehyde, 70% picric acid). The cervical spinal cord region was dissected out and post-fixed overnight at 4° C. in Bouin's post-fixative solution (9% formaldehyde, 70% picric acid). The following day the tissue was cryoprotected by immersion in 10% sucrose for 2 hours, 20% sucrose for 2 hours, and 30% sucrose overnight. The tissue was frozen in powdered dry ice, mounted in Tissue-Tek (Miles 4583) at −18° C., and 30 μm serial sagittal sections were mounted onto gel-subbed glass slides. Each slide also contained one or more 30 μm coronal sections through the lateral ventricles from the brain of the same animal to serve as a positive control. Slides were kept at −80° C. until processed. Immunohistochemistry: Slides were rinsed in PBS 3×15 minutes in 0.1M PBS at room temperature, hydrolyzed with 1N HCl for 60 minutes at 37° C., rinsed for 3×15 minutes in 0.1M PBS at room temperature, placed in 6% H_2O_2 in methanol for 30 minutes at room temperature, rinsed for 3×15 minutes in 0.1M PBS at room temperature, and incubated in 10% normal horse serum (Sigma H-0146) in 0.1M PBS or 20 minutes at room temperature. Slides were incubated overnight at room temperature in anti-BrdU monoclonal antibody (Becton 7580) that was diluted 1:50 in 0.1M PBS containing 1.5% normal horse serum and 0.3% TRITON®. The following day the slides were rinsed in PBS for 3×10 minutes in 0.1M PBS at room temperature, incubated with biotinylated horse anti-mouse IgG (Vector BA-2000) for 2 hours at room temperature, rinsed for 3×15 minutes in 0.1M PBS at room temperature, incubated in ABC reagent (Vector PK-6100) for 2 hours at room temperature, rinsed for 3×15 minutes in 0.1M PBS at room temperature, and developed with DAB reagent for 2 to 4 minutes. The slides were coverslipped with Aqua Polymount (Polysciences 18606). The number of BrdU positive cells was counted per cervical spinal cord section. Some BrdU labelled cells were found in the saline control sections. Treatment with either EGF or FGF-2 resulted in a significant increase in the number of BrdU labelled cells seen compared to control. The combination of EGF plus FGF-2 produced even a greater amount of BrdU positive cells per section.

EXAMPLE 31: In Vivo Infusion of Growth Factors into Ventricles to Increase Yield of Neural Stem Cells That Proliferate In Vitro

EGF pumps were implanted as described in Example 27. Animals were sacrificed by cervical dislocation 6 days after the pump was implanted. Brains were removed and the stem cells isolated and counted as described in Example 5.

Animals infused with EGF into the lateral ventricles for 6 days prior to sacrifice and brain culturing had 4 times as many spheres forming after 9 days in vitro compared to control animals which received saline pumps for the same 6 day period. Thus, infusing EGF into the lateral ventricles in vivo prior to removal and dissociation of neural tissue, greatly increases the yield of stem cells which proliferate and form neurospheres in vitro.

EGF and FGF can be infused into the ventricles to further increase the yield of neural stem cells obtainable from the neural tissue. Neurospheres generated by this method are used as a source of donor cells for later transplantation into degenerated areas of human adult CNS. Neurospheres can also be proliferated accordingly from a patient's own CNS stem cells and transplanted back into the patient.

EXAMPLE 32: In Vivo Modification of Neural Cells with bcl-2 Gene

A retroviral construct containing the human bcl-2 gene and the β-galactosidase gene is microinjected into the adult mouse lateral ventricle. A control mouse is injected with a retroviral construct containing only the β-galactosidase gene. One of the two progeny of each of the constitutively proliferating subependymal cells of the adult lateral ventricle normally dies within a few hours after division. The bcl-2 gene product prevents the programmed death of cells in several other tissues. In the adult subependyma, single cells infected with both the β-galactosidase and bcl-2 genes are marked by expression of both these gene products. These cells are identified in brain tissue slices with antibodies specific to β-galactosidase and human Bcl-2. Proliferating infected subependymal cells so infected produce larger numbers of cells per clone relative to the control. Thus, Bcl-2 induces the survival of the one normally dying progeny of each division of a constitutively proliferating adult subependymal cell. Moreover, the bcl-2 infected progeny migrate out into striatal and septal tissue to produce new neurons and glia. This indicates that EGF and Bcl-2 act as a survival factors for the normally dying progeny of constitutively proliferating adult subependymal cells which generate new neurons and glia in vivo.

EXAMPLE 33: In Vivo Modification of Neural Cells with NGF Gene

A retroviral construct containing the NGF gene is microinjected using the procedure described in Example 27 to infect the constitutively proliferating adult subependymal cells of the lateral ventricle. Thus, these cells are used to produce an endogenous growth factor in the adult brain. Levels of NGF produced by the transfected cells are measured directly by radioimmunoassay and (from a direct functional assay) by rescue of basal forebrain cholinergic neurons in vivo after axotomy injury in the model developed by Gage and collaborators (P.N.A.S. 83:9231, 1986).

EXAMPLE 34: Generation of Dopamine Cells in the Striatum by the Administration of a Composition Comprising Growth Factors to the Lateral Ventricle

Adult male CD, mice were anesthetized and placed in a stereotaxic apparatus. A cannula, attached to an ALZET minipump, was implanted into a lateral ventricle of each animal. The minipumps were subcutaneously implanted and were used to deliver (a) conditioned medium (from the rat B49 glial cell line, obtained from D Schubert, Salk Institute) plus bFGF (R&D Systems, 25 μg/ml) plus heparan sulfate (Sigma, 10 IU/ml) (CMF) or (b) EGF (Chiron, 25 μg/ml) plus bFGF (25 μg/ml) plus heparan sulfate (10 IU/ml) plus 25% FBS (E+F+FBS) or (c) sterile saline solution (SAL) as a control, into the lateral ventricles. Once batch of animals was sacrificed one day after completion of the delivery

regimen and the others were sacrificed twenty days later. The subventricular zones (SVZs) of these mice were dissected out, separating the cannulated, and therefore treated, side from the noncannulated control sides. The substantia nigra (SN) region of these mice were also recovered. Total RNA was extracted from these tissues using the guanidium thiocyanate acid phenol method [Chomzynski and Sacchi, *Annal. Biochem.* 162: 156–159, (1987)]. The RNA was then reverse transcribed to produce cDNA. These cDNAs were subject to PCR using primers designed to bracket a 254 nucleotide region of the TH messenger RNA (mRNA) and thermal cycling conditions favoring quantitative amplification. The PCR products were electrophoresed on a 2% agarose gel and then capillary blotted onto a positively charged nylon membrane. Radioactively labelled cDNA probe to TH was hybridized to the filter and detected by autoradiography. The autoradiograph was scanned and analyzed by densitometry to obtain relative levels of mRNA for TH in the SVZs of the cannulated sides in response to the treatments in the non-cannulated control SVZs and in the SN. In animals analyzed one day after treatment, the administration of E+F+FBS produced an eleven-fold increase in the level of TH mRNA in the SVZ compared to that observed in response to CMF, which in turn was more than twice the level seen with SAL. Twenty one days after treatment, the amount of TH mRNA detected in response to treatment with E+F+FBS was approximately the same as that detected after one day, while CMF and SAL treated SVZs had TH mRNA levels which were below detectable limits and were indistinguishable from the non-cannulated SVZ controls. Under all treatments, the SN had measurable amounts of TH mRNA.

EXAMPLE 35: Detection of Dopaminerzic Cells in Striatal TYssue Using Dual Labeing

Male CD, mice (Charles River, approximately 4 to 6 weeks old) were given intraperitoneal injections of BrdU (Sigma, 120 mg/kg) at 2 hour intervals over a 24 hour period, in order to label mitotically active cells. A cannula attached to an ALZET minipump was then implanted unilaterally into a lateral ventricle of each animal in order to deliver compositions a–c (CMF, E+F+FBS, or sterile saline) described in Example 34.

Animals were sacrificed 24 hours after the administration of growth factors using a lethal dose of pentobarbital anesthetic. The animals were then perfused through the heart with 10 ml of ice could 4% paraformaldehyde solution. The brains were removed and tissue in the region extending from the olfactory bulb to the third ventricle, including the striatum, was dissected out and stored overnight at 4° C. in a 30% sucrose/4% paraformaldehyde solution. The tissue was then frozen on dry ice and kept at −70° C. until processed. 30 μm coronal sections were cut using a cryostat and the sections were placed in 12 well porcelain dishes, to which 400 μl of PBS had been added. Sections were rinsed with fresh PBS and incubated overnight with the following primary antibodies: anti-TH (rabbit polyclonal, 1:1000, Eugene Tech International Inc.; or 1:100, Pel-freeze) and mouse anti-BrdU (1:55, Amersham), prepared in PBS/10% normal goat serum/0.3 TRITON®X-100. Following three rinses in PBS, goat anti-rabbit rhodamine and goat anti-mouse fluorescein (Jackson) were applied in PBS for 50 minutes at room temperature. Sections were then washed three times (10 minutes each) in PBS, placed on glass slides, dried and then coverslipped using Fluorsave (Calbiochem #345789).

The location of dopaminergic neurons was determined by mapping the location of TH-immunoreactive (TH+) cells, or TH+ and BrdU+ cells in relation to the ventricles. In response to saline injections made into the lateral ventricles, the normal population of TH+ fibers were detected in the striatum but no TH+ cell bodies were detected in this region. CMF treatment resulted in the detection of TH+ cell bodies, in addition to the normal population of TH+ fibers, in the striatum and in the region of the third ventricle. E+F+FBS treatment had the most profound effect resulting in the detection of the most TH+ cell bodies. Several of the TH+ cell bodies were also BrdU positive.

EXAMPLE 36: Rat Model of Parkinson's Disease Measures the Effects of In Vivo Administration of Growth Factors

The 6-OHDA lesion rat model of Parkinson's disease is used to measure the effects of administering various combinations of growth factors to the lateral ventricle. Unilateral 6-OHDA lesions are performed in the rat model and rotation behavior is observed. Minipumps are subcutaneously implanted into the animals as described in Example 34. EGF (Chiron, 25 μg/ml) plus bFGF (25 μg/ml) plus heparan sulfate (10 IU/ml) plus 25% FBS is continuously administered to the lateral ventricle. Saline is administered to control animals. The ability to overcome the rotational bias produced by the unilateral 6-OHDA lesions is observed.

EXAMPLE 37: Screening of Drugs or Other Biological Agents for Effects on Multipotent Neural Stem Cells and Neural Stem Cell Progeny

A. Effects of BDNF on Neuronal and Glial Cell Differentiation and Survival

Precursor cells were propagated as described in Example 4 and differentiated using Paradigm 3 described in Example 7. At the time of plating the EGF-generated cells, BDNF was added at a concentration of 10 ng/ml. At 3, 7, 14, and 21 days in vitro (DIV), cells were processed for indirect immunocytochemistry. BrdU labeling was used to monitor proliferation of the precursor cells. The effects of BDNF on neurons, oligodendrocytes and astrocytes were assayed by probing the cultures with antibodies that recognize antigens found on neurons (MAP-2, NSE, NF), oligodendrocytes (O4, GalC, MBP) or astrocytes (GFAP). Cell survival was determined by counting the number of immunoreactive cells at each time point and morphological observations were made. BDNF significantly increased the differentiation and survival of neurons over the number observed under control conditions. Astrocyte and oligodendrocyte numbers were not significantly altered from control values.

B. Effects of BDNF on the Differentiation of Neural Phenotypes

Cells treated with BDNF according to the methods described in Part A were probed with antibodies that recognize neural transmitters or enzymes involved in the synthesis of neural transmitters. These included TH, ChAT, substance P, GABA, somatostatin, and glutamate. In both control and BDNF-treated culture conditions, neurons tested positive for the presence of substance P and GABA. As well as an increase in numbers, neurons grown in BDNF showed a dramatic increase in neurite extension and branching when compared with control examples.

C. Identification of Growth-Factor Responsive Cells

Cells that are responsive to growth factor treatment were identified by differentiating the EGF-generated progeny as

described in Example 7, paradigm 3 and at 1 DIV adding approximately 100 ng/ml of BDNF. At 1, 3, 6, 12 and 24 hours after the addition of BDNF the cells were fixed and processed for dual label immunocytochemistry. Antibodies that recognize neurons (MAP-2, NSE, NF), oligodendrocytes (O4, GalC, MBP) or astrocytes (GFAP) were used in combination with an antibody that recognizes c-fos and/or other immediate early genes. Exposure to BDNF results in a selective increase in the expression of c-fos in neuronal cells.

D. Effects of BDNF on the Expression of Markers and Regulatory Factors During Proliferation and Differentiation

Cells treated with BDNF according to the methods described in Part A are processed for analysis of the expression of FGF-R1, as described in Example 39 or other markers and regulatory factors, as described in Example 40.

E. Effects of BDNF administration During Differentiation on the Electrophysiological Properties of Neurons

Neurons treated with BDNF during differentiation, according to the methods described in Part A, are processed for the determination of their electrophysiological properties, as described in Example 41.

F. Effects of Chlorpromazine on the Proliferation, Differentiation, and Survival of Growth Factor Generated Stem Cell Progeny

Chlorpromazine, a drug widely used in the treatment of psychiatric illness, is used in concentrations ranging from 10 ng/ml to 1000 ng/ml in place of BDNF in Examples 7A to 7E above. The effects of the drug at various concentrations on stem cell proliferation and on stem cell progeny differentiation and survival is monitored. Alterations in gene expression and electrophysiological properties of differentiated neurons are determined.

EXAMPLE 38: Stem Cell Proliferation Assay

Primary cells were obtained from E14 mice and prepared as detailed in Examples 1 and 4. Either EGF, EGF and FGF or EGF and BMP-2 were added to complete medium at a concentration of 20 ng/ml of each growth factor, with the exception of BMP-2 which was added at a concentration of 10 ng/ml. Cells were diluted with one of the prepared growth factor-containing media to a concentration of 25,000 cells/ml. 200 μl of the cell/medium combination were pipetted into each well of a 96-well place (Nuclon) with no substrate pretreatment. Cells were incubated under the same conditions as outlined in Example 4.

After 8–10 DIV the number of neurospheres was counted and the results tabulated. As cells grown in a combination of EGF and FGF produced significantly more neurospheres than cells grown in the presence of EGF alone. The combination of EGF and BMP-2 inhibited neurosphere development.

EXAMPLE 39: Comparison of Receptor and Growth Factor Expression in Undifferentiated vs. Differentiated Stem Cell-Derived Progeny by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Neurospheres were generated as described in Example 4, and some were differentiated as per Paradigm 1, Example 7. RNA from either undifferentiated or differentiated neurospheres was isolated according to the guanidinium thiocyanate acid phenol procedure of Chomzynski and Sacchi *Anal. Biochem.* 162: 156–159 1987)]. Complementary DNA (cDNA) was synthesized from total RNA using reverse transcriptase primed with oligo dT. Gene-specific primers were designed and synthesized and these primers were used in PCR to amplify cDNAs for different growth factors and growth factor receptors. Amplified material was run on agarose gels alongside molecular weight markers to ensure that PCR products were of the expected size, while the identity of PCR fragments was confirmed by restriction enzyme analysis and by sequencing [Arcellana-Panlilio, *Methods Enzymol.* 225: 303–328 (1993)]. An ethidium-stained agarose gel visualized via UV transillumination showed the detection of three growth factor receptor transcripts, namely EGF-R, FGF-R, and LIF-R, in undifferentiated and differentiated stem cell-derived progeny. Table I lists the primer sets analyzed and the results of undifferentiated and differentiated cells.

TABLE I

Primer Sets Analyzed	Undifferentiated Cells	Differentiated Cells
Actin	+	+
NGF	+	nd
EGFr[m]	+	+
bFGFr	+	+
LIFr[m]	+	+
tyrosine hydroxylase	+	+
choline acetyltransferase[m]	nd	+
cholecystokinin[m]	nd	−
enkephalin[m]	nd	+
tyrosine kinase-rA	+	+
tyrosine kinase-rB	+	+++++
tyrosine kinase-rC	+	+

r = receptor
m = derived from mouse
nd = no data available

EXAMPLE 40: Isolation of Novel Markers and Regulatory Factors Involved in Neural Stem Cell Proliferation and Differentiation

Neurospheres are generated as described in Example 4 using CNS tissue from CD, albino mice (Charles River). Some of these neurospheres are allowed to differentiate according to the rapid differentiation paradigm of Example 7 producing cultures enriched in neurons, astrocytes, and oligodendrocytes. Total RNA is extracted from the undifferentiated neurospheres as well as the differentiated cell cultures using the guanidinium thiocyanate acid phenol method referred to in Example 39. Messenger RNA (mRNA) is isolated by exploiting the affinity of its poly A tract to stretches of either U's or T's. Reverse transcription of the mRNA produced cDNA, is then used to make primary libraries in either plasmid [Rothstein et al., *Methods in Enzymology* 225:587–610 (1993)] or lambda phage vectors. To isolate cDNAs that are specific to either undifferentiated or differentiated stem cell derived progeny, cDNA from one is hybridized to RNA from the other, and vice versa. The unhybridized, and thus culture type-specific, cDNAs in each case are then used to construct subtracted libraries [Lopez-Fernandez and del Mazo, *Biotechnigues* 15(4):654–658 (1993)], or used to screen the primary libraries.

Stem cell-derived undifferentiated cell specific and differentiated cell specific cDNA libraries provide a source of

clones for novel markers and regulatory factors involved in CNS stem cell proliferation and differentiation. Specific cDNAs are studied by sequencing analysis to detect specific sequence motifs as clues to identity or function, and database searching for homologies to known transcripts. Using cDNAs in a hybridization to various RNA samples electrophoresed on an agarose-formaldehyde gel and transferred to a nylon membrane, allows the estimation of size, relative abundance, and specificity of transcripts. All or portions of cDNA sequences are used to screen other libraries in order to obtain either complete mRNA sequences or genomic sequence information. Antibodies directed against fusion proteins generated from specific cDNAs are used to detect proteins specific to a particular cell population, either by immunocytochemistry or by Western Blot analysis. Specific gene sequences are used to isolate proteins that interact with putative regulatory elements that control gene expression. These regulatory elements are then used to drive the expression of an exogenous gene, such as beta-galactosidase.

EXAMPLE 41: Electrophysiological Analysis of Neurons Generated From Growth Factor-Responsive Stem Cells and Exposed to a Biological Agent

Neurospheres were generated as described in Example 4. Neurospheres were dissociated using the technique described in paradigm 2, Example 7. The clonally derived cells were plated at low density and differentiated in the presence of bFGF. The electrophysiological properties of cells with the morphological appearance of neurons were determined as described as described by Vescovi et al. [*Neuron*, 11: 951–966 (1993)]. Under whole cell current clamp, the mean resting potential and input resistance were −62±9 mV and 372±MΩ. Rectangular suprathreshold current steps, (~100 pA) elicited regenerative potential responses in which the amplitude and time course were stimulus dependent. After the completion of electrophysiological experiments, the cell morphology was visualized by intracellular excitation of 5-carboxyfluorescein.

EXAMPLE 42: Screening for the Effects of Drugs or Other Biological Agents on Growth Factor-Responsive Stem Cell Progeny Generated From Tissue Obtained From a Patient with a Neurological Disorder

The effects of BDNF on the EGF-responsive stem cell progeny generated from CNS tissue obtained at biopsy from a patient with Huntington's disease is determined using the methods outlined in Example 7, A to E. BDNF is a potent differentiation factor for GABAergic neurons and promotes extensive neuronal outgrowth. Huntington's Disease is characterized by the loss of GABAergic neurons (amongst others) from the striatum.

EXAMPLE 43: Assay of striatum-derived neurosphere proliferation in response to various combinations of proliferative and regulatory factors

Paradigm 1: Primary striatal cells prepared as outlined in Example 1 were suspended in Complete Medium, without growth factors, plated in 96 well plates (Nunclon) and incubated as described in Example 4. Following a one hour incubation period, a specific proliferative factor, or a combination of proliferative factors including EGF, or bFGF (recombinant human bFGF: R & D Systems), or a combination of EGF and bFGF, or EGF plus FGF plus heparan sulfate (Sigma), or bFGF plus heparan sulfate made up in Complete Medium at a concentration of 20 ng/ml for each of the growth factors and 2 μg/ml for heparan sulfate), was added to each well of the plate.

Activin, BMP-2, TGF-β, IL-2, IL-6, IL-8, MIP-1α, MIP-1β, MIP-2 (all obtained from Chiron Corp.), TNFα, NGF (Sigma), PDGF (R&D Systems), EGF and CNTF (R. Dunn and P. Richardson, McGill University) were made up in separate flasks of compete medium to a final concentration of 0.2 μg/ml. Retinoic acid (Sigma) was added at a concentration of 10^{-6}M. 10 μl of one of these regulatory factor-containing solutions was added to each proliferative factor-containing well of the 96 well plates. Control wells, containing only proliferative factors, were also prepared.

In another set of experiments, the neurosphere inducing properties of each of these regulatory factors was tested by growing cells in their presence, in proliferative factor-free Complete Medium. None of these regulatory factors, with the exception of EGF, when used in the absence of a proliferation-inducing factor such as EGF or FGF, has an effect on neural stem cell proliferation.

The activin, BMP-2, TGF-μ, IL-2, IL-6, IL-8, MIP-1α, MIP-1β, MIP-2, TNFα and EGF additions were repeated every second day, CNTF which was added each day and retinoic acid, NGF and PDGF were added only once, at the beginning of the experiment. The cells were incubated for a period of 10–12 days. The number of neurospheres in each well was counted and the resulting counts tabulated using Cricket Graph III. Other relevant information regarding sphere size and shape were also noted.

In general, bFGF had a greater proliferative effect than EGF on the numbers of neurospheres generated per well. In the presence of 20 ng/ml EGF, approximately 29 neurospheres per well were generated. In the presence of bFGF, approximately 70 neurospheres were generated. However, in bFGF alone, the neurospheres were only about 20% of the size of those generated in the presence of EGF. The combination of EGF and bFGF produces significantly more neurospheres than does EGF alone, but fewer than seen with bFGF alone. The neurospheres are larger than those seen in bFGF alone, approximating those seen in EGF. In the case of bFGF generated spheres, the addition of heparan sulfate increased the size of the spheres to about 70% of the size of those which occur in response to EGF. These data suggest that EGF and FGF have different actions with respect to the induction of stem cell mitogenesis.

The effects of the regulatory factors added to the proliferative factor-containing wells are summarized in Table II. In general, the TGFβ family, interleukins, macrophage-inhibitory proteins, PDGF, TNFα, retinoic acid (10^{-6}M) and CNTF significantly reduced the numbers of neurospheres generated in all of the proliferative factors or combinations of proliferative factors tested. BMP-2 (at a dose of 10 ng/ml), completely abolished neurosphere proliferation in response to EGF. EGF and heparan sulfate both greatly increased the size of the neurospheres formed in response to bFGF (about 400%).

TABLE II

	PROLIFERATIVE FACTORS									
	EGF		bFGF		EGF + bFGF		bFGF + Heparan		EGF + bFGF + Heparan	
REGULATORY FACTORS	#	size	#	size	#	size	#	size	#	size
TGFβ Family♦	−57%	–	−57%	–	−34%	—	−55%	–	−20%	–
BMP-2	−100%	n/a	−5%	=	+16%	—	−3%	–	+10%	—
Interleukins	−21%	=	−23%	=	−37%	–	−28%	=	−39%	–
MIP Family	−25%	=	−6%	=	−32%	–	−22%	=	−33%	–
NGF	−10%	=	0%	=	−30%	=	+5%	=	−48%	=
PDGF	−1.5%	=	−4%	=	−26%	=	−10%	=	−27%	=
TNFα	−17%	=	−17%	=	−41%	=	−21%	=	−37%	=
10^{-6}M Retinoic Acid	−8%	—	−61%	–	−31%	—	−65%	—	−45%	—
CNTF	−23%	–	−77%	¯	−81%	—	−81%	–	−84%	—
EGF	–		−14%	++	–		−17%	=	–	
Heparan Sulfate	0%	=	0%	++	0%	=				

♦Excluding BMP-2 (i.e. TGFα and activin)
Numbers of neurospheres generated (#) are given as percentages that reflect the decrease (−) or increase (+) in numbers of neurospheres per well, in response to a PROLIFERATIVE FACTOR in the presence of a REGULATORY FACTOR, compared with the number of neurospheres proliferated in the absence of the REGULATORY FACTOR.
Size of neurospheres generated in the presence of PROLIFERATIVE FACTORS and REGULATORY FACTORS compared to those generated in the presence of PROLIFERATIVE FACTORS alone are indicated as follows: ++: much larger; +: larger; =: approximately the same size; ¯: variable in size; –: smaller; —: much smaller

Antisense and sense experiments were carried out using the following oligodeoxynucleotides (all sequences written 5' to 3'):

EGF receptor:	Sense strand:	GAGATGCGACCCTCAGGGAC (SEQ ID NO: 1)
	Antisense strand:	GTCCCTGAGGGTCGCATCTC (SEQ ID NO: 2)
EGF:	Sense strand:	TAAATAAAAGATGCCCTGG (SEQ ID NO: 3)
	Antisense strand:	CCAGGGCATCTTTTATTTA (SEQ ID NO: 4)

Each oligodeoxynucleotide was brought up and diluted in ddH_2O and kept at −20° C. Each well of the 96 well plates received 10 μl of oligodeoxynucleotide to give a final concentration of either 1, 2, 3, 4, 5, 10 or 25 μM. Oligodeoxynucleotides were added every 24 hours. The EGF receptor (EGFr) and EGF oligodeoxynucleotides were applied to cultures grown in bFGF (20 ng/ml), and EGFr oligodeoxynucleotides were applied to cultures grown in EGF (20 ng/ml). Cells were incubated at 37° C., in a 5% CO_2 100% humidity incubator. After a period of 10 to 12 days, the number of neurospheres per well was counted and tabulated. A concentration of 3 μM of antisense oligodeoxynucleotides produced a 50% reduction in the number of neurospheres generated per well, whereas the sense oligodeoxynucleotides had no effect on the number of neurospheres generated in response to EGF and FGF. Both the sense and antisense oligodeoxynucleotides were toxic to cells when 10 μM or higher concentrations were used.

Similar experiments can be performed using the following oligonucleotides:

FGF receptor:	Sense strand:	GAACTGGGATGTGGGGCTGG (SEQ ID NO: 5)
	Antisense strand:	CCAGCCCCACATCCCAGTTC (SEQ ID NO: 6)
FGF:	Sense strand:	GCCAGCGGCATCACCTCG (SEQ ID NO: 7)

-continued

Antisense strand:	CGAGGTGATGCCGCTGGC (SEQ ID NO: 8)

The FGF receptor (FGFr) and FGF oligodeoxynucleotides are applied to cultures grown in EGF, and FGFr oligodeoxynucleotides are applied to cultures grown in bFGF.

Paradigm 3: Embryonic tissue is prepared as outlined in Example 1 and plated into 96 well plates. Complete Medium, containing 20 ng/ml of either EGF of bFGF is added to each well. 10 μyl of diluted phorbol 12-myristate 13 acetate (PMA) is added once, at the beginning of the experiment, to each well of the 96 well plates, using an Eppendorf repeat pipetter with a 500 μl tip to give a final concentration of either 10, 20, 40, 100 or 200 μg/ml. Cells are incubated at 37° C. in a 5% CO_2 100% humidity incubator. After a period of 10 to 12 days the number of neurospheres per well is counted and tabulated.

Paradigm 4: Embryonic tissue is prepared as outlined in Example 1 and plated into 96 well plates. 10 μl of diluted staurosporine is added to each well of a 96 well plate, using an Eppendorf repeat pipetter with a 500 μl tip to give a final concentration of either 10, 1, 0.1, or 0.001 μM of staurosporine. Cells are incubated at 37° C., in a 5% CO_2 100% humidity incubator. After a period of 10 to 12 days, the number of neurospheres per well is counted and tabulated.

EXAMPLE 44: Adult spinal cord stem cell proliferation—in vitro responses to specific biological factors or combinations of factors

Spinal cord tissue was removed from 6 week to 6 month old mice, as follows: cervical tissue was removed from the vertebral column region rostral to the first rib; thoracic spinal tissue was obtained from the region caudal to the first rib and approximately 5 mm rostral to the last rib; lumbar-sacral tissue constituted the remainder of the spinal cord. The dissected tissue was washed in regular artificial cerebrospinal fluid (aCSF), chopped into small pieces and then placed into a spinner flask containing oxygenated aCSF with high Mg^{2+} and low Ca^{2+} and a trypsin/hyaluronidase and

kynurenic acid enzyme mix to facilitate dissociation of the tissue. The tissue was oxygenated, stirred and heated at 30° C. for 1½ hours, then transferred to a vial for treatment with a trypsin inhibitor in media solution (DMEM/12/hormone mix). The tissue was triturated 25–50 times with a fire narrow polished pipette. The dissociated cells were centrifuged at 400 r.p.m. for 5 minutes and then resuspended in fresh media solution. Cells were plated in 35 mm dishes (Costar) and allowed to settle. Most of the media was aspirated and fresh media was added. EGF alone, or EGF and bFGF were added to some of the dishes to give a final concentration of 20 ng/ml each, and bFGF (20 ng/ml) was added, together with 2 μg/ml of heparan sulfate, to the remainder of the dishes. The cells were incubated in 5% CO_2, 100% humidity, at 37° C. for 10–14 days. The numbers of neurospheres generated per well were counted and the results tabulated. EGF alone resulted in the generation of no neurospheres from any of the spinal cord regions. In the presence of EGF plus bFGF, neurospheres were generated from all regions of the spinal cord, in particular the lumbar sacral region. The combinations of EGF+FGF and FGF+ heparan sulfate produced similar numbers of spheres in the cervical region, whereas the combination of bFGF plus heparan sulfate resulted in fewer neurospheres from the thoracic and lumbar regions.

EXAMPLE 45: Transplantation of Multipotent Neural Stem Cell Progeny in Animal Models

I. TRANSPLANTATION PROCEDURE

1. Neurosphere preparation

Neural tissue was obtained from normal embryonic or adult CD1 mice and from embryonic or adult Rosa 26 mice (transgenic animals derived from C57/BL/6 mice, which express the β-galactosidase gene in all cells, thus allowing the transplanted cells to be easily detected in host tissue). Neurospheres were generated using the procedures described in Examples 1–5, passaged 2 to 8 times (see Example 6), and maintained in culture for 6–10 days after the last passage.

2. Labeling and Preparation of Neural Stem Cell Progeny

16 hours prior to transplantation, neurospheres derived from embryonic and adult tissue were labeled with BrdU by adding BrdU to the media for a total concentration of 1 μM and/or with fluorescent latex beads (Polysciences; 1:100 dilution of 0.75 μm beads). Neurospheres were detached from the substrate by gentle shaking, poured into 50 ml centrifuge tubes and spun down (5 minutes, 400 r.p.m., 15° C., no brake) to remove the proliferation-inducing media used for the proliferation culture. The neurospheres derived from embryonic tissue were then washed twice in Hank's buffered salt solution (HBSS), resuspended in 2 ml HBSS and dissociated by trituration (spheres drawn into a fire-polished pasteur pipette 40×). The neurospheres derived from adult tissue were trypsinized (0.05% in EDTA media; 5–10 min) and then a trypsin inhibitor (ovomucoid; 0.7–1.0 mg/ml in media) was added. The tubes were swirled and the neurospheres were recentrifuged (400 r.p.m., 15° C., no brake). Cells were resuspended in 2 ml media (DMEM F12/hormone mix) and dissociated by mechanical trituration (25×).

Live and dead cells obtained from neurospheres derived from embryonic and adult tissue were counted prior to being centrifuged to remove dead cells (10 min., 400 r.p.m., 15° C., no brake). The live cells were resuspended to appropriate cell density ($1–50×10^6$ cells/ml). The cells were recounted to determine the number of live and dead cells and cell viability was calculated. The cells were then transferred to a microcentrifuge tube for storage on ice prior to transplantation. When ready for use, cells were resuspended prior to each cell injection by drawing cells into an eppendorf pipette tip (200 or 1000 μl).

3. Transplantation of Neural Stem Cell Progeny

The donor neural stem cell progeny were transplanted into selected sites in the brain of normal, healthy neonate or adult CD1 or C57BL/6 mice or adult Wistar or Sprague-Dawley rats. In some cases, embryonic cells from CD1 mice received in vitro gene transfer procedures prior to transplantation of the cells. The host animals were anaesthetized with sodium pentobarbital (65 mg/Kg) and placed into a stereotaxic apparatus. A skin incision was made to expose the surface of the skull or vertebrae. Injection sites were located using stereotaxic coordinates to locate the desired site. Burr holes were drilled in the skull and vertebrae at the coordinate sites. A 5 μl syringe was housed on a syringe pump and attached to a stainless steel cannula (30–31 gauge) via a short length of polyethylene tubing. A small air bubble and then 4–5 μl of the desired cell suspension was drawn into the cannula. The cannula was lowered to the desired location and 1–3 μl of the cell suspension was injected at a speed of 0.1–0.5 μl/min. Animals that received xenografts or allograft were treated with 0.1 mg/ml cyclosporin A in the drinking water to reduce the risk of tissue rejection.

4. Analysis of Transplanted Neural Stem Cell Progeny

The animals were allowed to survive for 2–12 weeks prior to sacrifice. At a specified time after transplantation, animals were perfused transcardially for aldehyde fixation of the brain and spinal cord tissue. A low-high pH perfusion protocol was used (Sloviter & Nilaver, (1987) *Brain Res.* Vol. 330:358–363). After perfusion, brains and spinal cords were removed, post-fixed, and then cryoprotected in sucrose/PBS for cutting in a cryostat. Sections of tissue (10 μM) were cut and mounted on microscope slides directly in a sequential way so that adjacent sections could be examined with different anatomical protocols.

Survival of transplanted cells labeled with fluorescent beads were identified by the localization of fluorescent beads within the cell cytoplasm. BrdU labeled cells (cells that had incorporated BrdU into their DNA during cell division in culture prior to transplantation) were identified using antibodies against BrdU (1:250–500; Monoclonal-Sera-lab; Polyclonal-Accurate Chem. & Sci). Antibodies against GFAP (1:250 Monoclona-Boehringer, Polyclonal-BTI), or NeuN (1:250–500; Monoclonal-R.J. Mullen) were then used to identify the differentiation of the transplanted cells. Cell transplants derived from transgenic animals expressing β-galactosidase were histochemically analyzed using methodology described by Turner and Cepko (1987) (*Nature* 328:131–136)and by immunohistochemical staining. For Rosa 26 cells, antibodies against β-galactosidase were used to identify the transplanted cells and antibodies to NeuN were used to identify cells that had differentiated into neurons. Human cells were identified with HLA antibodies (1:250, Monoclonal-Sera-labs). Antibodies were incubated with the tissue samples and detected using standard immunohistochemical protocols.

The results obtained from the animal models described below are summarized in Tables II–V.

A. MODEL OF HUNTINGTON'S DISEASE

Rats were anesthetized with nembutal (25 mg/kg i.p) and injected with atropine sulfate (2 mg/kg i.p.). Animals sustained an ibotenate lesion of the striatum, stimulating Huntington's Disease in the animals. 7 days after the lesion, the

animals received an injection of cells prepared as in Examples 1–5 under stereotaxic control. Injections were made to the lesioned area via a 21-gauge cannula fitted with a teflon catheter to a microinjector. Injected cells were labelled with fluorescein-abelled microspheres. Animals were given behavioral tests before the lesion, after the lesion, and at various intervals after the transplant to determine the functionality of the grafted cells at various post-operative time points, then killed and perfused transcardially with 4% buffered paraformaldehyde, 0.1% glutaraldehyde and 5% sucrose solution at 4° C. The brains were frozen in liquid nitrogen and stored at −20° C. until use. Brains sections were sliced to 26 μm on a cryostat, fixed in 4% paraformaldehyde and stained using the M6 monoclonal antibody to stain for mouse neurons, a nd then reacted with a secondary anti-rat fluorescein-conjugated antibody. Neuronal and glial phenotype was identified by dual labeling of the cells with antibody to NSE and GFAP.

B. PARKINSON'S DISEASE

Two animal models of Parkinson's Disease were used. In the first model, unilateral dopamine neurons of the substantia nigra were lesioned by the stereotaxic administration of 6-OHDA into the substantia nigra in adult CD 1 (1–4 μg) and C57BL/6 mice (1 μg), and Wistar rats (16 μg). Mice were pretreated with desipramine (25 mg/Kg i.p.) and rats were pretreated with pargyline (50 mg/Kg i.p.) both of which prevent the action of 6-OHDA on noradrenergic neurons and allow the selective destruction of dopaminergic neurons. In one series of experiments, multipotent neural stem cell proge ny obtained from embryonic Rosa 26 mice, were prepared using the procedures described in Examples 1 and 4. The neural stem cell progeny were labeled, prepared, and transplanted into the striatum of the lesioned C57BL/6 mice using the methods described above in this Example. In a second series of experiments, the cells were administered to the same regions in the brains of adult 6-OHDA Wister rats. In a third series of experiments, proliferated fetal human cells (prepared as outlined in Example 9), were transplanted into the striatum of the 6-OHDA lesioned CD1 mice. After a survival period of 2 weeks, the host animals were sacrificed and their brains removed. The brain tissue was treated and analyzed as described above. The second animal model used was the adult mutant Weaver mice (Jackson Labs, 3-½ months), in which approximately 70% of the dopaminergic neurons of the substantia nigra are lost by the age of 3 months. Animals were anaesthetized and the proliferated progeny of multipotent neural stem cells derived from embryonic Rosa 26 mice were injected into the striatal region of the animals according to the methods described above. The animals were allowed to survive for 15 days prior to sacrifice and analysis of striatal tissue.

C. CARDIAC ARREST

Transient forebrain ischemia was induced in adult Wistar rats by combining bilateral carotid occlusion with hypovolemic hypotension (Smith et al. (1984) *Acta Neurol Scand* 69:385–401). These procedures lesion the CA1 hippocampal pyramidal cells which is typical of damage observed in humans following cardiac arrest and the cause of severe memory and cognitive deficits. The progeny of proliferated multipotent neural stem cells, derived from embryonic Rosa 26 mice, were prepared as described above and transplanted into the lesioned hippocampal region of the ischemia lesioned rats. After 8 days, the animals were sacrificed and their brains were removed and analyzed. β-gal positive cells, indicating surviving cells from the Rosa 26 donor) were detected in the lesioned hippocampal region. In addition, double labeled β-gal/NeuN$^+$ cells were found indicating that transplanted cells had differentiated into neurons.

D. STROKE

Occlusion of the carotid arteries precipitates the occurrence of ischemic damage similar to that which occurs during stroke. Adult Wistar rats, in which the middle cerebral artery has been occluded to produce symptomatic lesions in the caudal striatum and parietal cortex, have neural stem cell progeny implanted into the lesioned areas. After a survival period, the animals are tested for behavioral improvements and are then sacrificed and their brains analyzed.

E. EPILEPSY

Implantation of an electrode into the amygdala is used to kindle the brain, inducing epileptic episodes and other symptoms of epilepsy. Neural stem cell progeny are transplanted into the hippocampal region. The animals are later tested for epileptic episodes and then sacrificed for analysis of the grafted tissue.

F. ALZHEIMER'S DISEASE

Cognitive impairment is induced in rats and mice by ibotenic acid lesions of the nucleus basalis, or old animals, exhibiting signs of dementia, are used. Neural stem cell progeny are transplanted into the frontal cortex, medial septal nucleus and the nucleus of the diagonal band of the brains of the animals. After a survival period, the animals are tested for cognitive ability and are then sacrificed to allow analysis of brain tissue.

G. SPINAL CORD INJURY AND DISEASE

Spasticity is a debilitating motor disorder that is a common consequence of disorders such as spinal cord injury, MS, and cerebral palsy. Transection of the spinal cord is used to produce muscular paralysis and is followed by the development of spasticity, which is characterized by debilitating hyperactive tendon reflexes, clonus and muscle spasms. Neural stem cell progeny are prepared and are transplanted into the lumbar lateral funiculus. After a survival period, the animals are examined for improvement in motor control and are then sacrificed to allow for analysis of spinal tissue.

TABLE III

DONOR CELL SOURCE	HOST	TRANSPLANT REGION	BrdU	BrdU/ GFAP	BrdU/ NeuN
Embryonic CD1 Mouse	Neonate CD1 Mouse	striatum	+	+	+
		frontal cortex	+	+	+
	Adult CD1 Mouse	striatum	+	+	+
		hippocampus	+	+	+
		frontal cortex	+	+	+
		parietal cortex	+	+	+
		MS/NDB	+	+	+
	Adult Wistar Rat	spinal cord	+	+	+
		hippocampus	+	+	+
		parietal cortex	+	+	
Adult CD1 Mouse	Adult CD1 Mouse	striatum	+	+	
		hippocampus	+		
		frontal cortex	+		
	Adult Wistar Rat	spinal cord	+		

TABLE IV

Donor Cell Source	HOST	Transplant Region	β-Gal	BrdU	BrdU/ GFAP
Embryonic CD1 Mouse	Adult CD1 Mouse	hippocampus	+	+	+
		frontal cortex	+	+	+

TABLE IV-continued

Donor Cell Source	HOST	Transplant Region	β-Gal	BrdU	BrdU/ GFAP
in vitro gene		parietal cortex	+	+	+
transfer		striatum	+	+	+
		MS/NDB	+	+	+
Embryonic	Adult DC1	striatum	+	+	+
Rosa	Mouse	parietal cortex	+	+	+
		MS/NDB	+	+	+
Adult Rosa 26	Adult C57/	hippocampus	+		
	BL/6 Mouse	frontal cortex	+		
		MS/NDB	+		

TABLE V

DONOR CELL SOURCE	HOST	β-Gal	BrdU/GFAP
Embryonic Rosa 26 Mouse	Adult 6-OHDA lesioned C57BL/6 mouse (striatal injections)	+	+
	Adult 6-OHDA lesioned Wistar rat (striatal injections)	+	+
Embryonic Rosa 26 Mouse	Adult Mutant Weaver Mouse (striatal injections)	+	+

All references, patents, and patent applications cited herein are incorporated herein by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 8

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAGATGCGAC CCTCAGGGAC 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTCCCTGAGG GTCGCATCTC 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TAAATAAAAG ATGCCCTGG 19

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCAGGGCATC TTTTATTTA 19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAACTGGGAT GTGGGGCTGG 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCAGCCCCAC ATCCCAGTTC 20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCCAGCGGCA TCACCTCG 18

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGAGGTGATG CCGCTGGC 18

What is claimed is:

1. A method of producing genetically modified neural cells comprising:

(a) obtaining cells derived from mammalian neural tissue containing at least one multipotent neural stem cell capable of producing progeny that are capable of differentiating into neurons, astrocytes, and oligodendrocytes, wherein said obtained cells have not been cultured in a serum-containing medium;

(b) preparing a substantially serum-free culture medium containing one or more predetermined growth factors capable of inducing multipotent neural stem cell proliferation, and

(c) preparing a cell culture by combining the cells obtained in (a) with the culture medium prepared in (b) to induce proliferation of said multipotent neural stem cell to produce proliferating multipotent neural stem cells and proliferating multipotent neural stem cell progeny which includes daughter multipotent neural stem cells and introducing exogenous DNA that does not contain oncogenes to genetically modify said proliferating multipotent neural stem cells and/or said multipotent neural stem cell progeny to produce genetically modified neural stem cell progeny.

2. The method of claim 1 wherein the growth factor in the culture medium prepared in (b) is selected from the group consisting of acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, amphiregulin, transforming growth factor alpha, and combinations thereof.

3. The method of claim 1 wherein said growth factor in the culture medium prepared in (b) is epidermal growth factor or transforming growth factor alpha.

4. The method of claim 1 wherein said growth factor in the culture medium prepared in (b) is a fibroblast growth factor.

5. The method of claim 4 wherein said culture medium prepared in (b) additionally contains epidermal growth factor.

6. The method of claim 1 wherein the growth factor in the culture medium prepared in (b) is epidermal growth factor.

7. The method of claim 1 wherein the culture medium prepared in (b) is defined.

8. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to produce a growth factor product.

9. The method of claim 8 wherein said growth factor product is selected from the group consisting of nerve growth factor, brain-derived neurotrophic factor, neurotrophins, ciliary neuroatrophic factor, amphiregulin, basic fibroblast growth factor, acidic fibroblast growth factor, epidermal growth factor, transforming growth factor-alpha, transforming growth factor-beta, platelet-derived growth factor, insulin-like growth factors, and interleukins.

10. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to produce a neuropeptide.

11. The method of claim 10 wherein said neuropeptide is selected from the group consisting of substance-P, neuropeptide-γ, enkephalin, vasopressin, vasoactive intestinal peptide, cholecystokinin, glucagon, bombesin, somatostatin, and calcitonin gene-related peptide.

12. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to express a growth factor receptor.

13. The method of claim 12 wherein said growth factor receptor is selected from the group consisting of low affinity nerve growth factor receptor, ciliary neurotrophic factor receptor, neurotrophin receptors, epidermal growth factor receptor, fibroblast growth factor receptor, and amphiregulin receptor.

14. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to express a neurotransmitter.

15. The method of claim 14 wherein said neurotransmitter is selected from the group consisting of serotonin, L-dopa, dopamine, norepinephrine, epinephrine, tachykinin, endorphin, histamine, N-methyl D-aspartate, glycine, glutamate, γ-amino butyric acid, and acetylcholine.

16. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to contain a neurotransmitter synthesizing gene.

17. The method of claim 16 wherein said neurotransmitter synthesizing gene is selected from the group consisting of tyrosine hydroxylase, dopa decarboxylase, dopamine-β-hydroxylase, phenylethanolamine N-methyltransferase, glutamic acid decarboxylase, tryptophan hydroxylase, choline acetyltransferase, and histidine decarboxylase.

18. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to express a neurotransmitter receptor.

19. The method of claim 1 wherein said multipotent neural stem cell progeny are genetically modified to express chromaffin granule amine transporter.

20. The method of claim 1 wherein the multipotent neural stem cell progeny produced in (c) grow in the form of clonally-derived clusters of cells.

21. The method of claim 1 wherein prior to genetically modifying said proliferating multipotent neural stem cell progeny, a subsequent cell culture is prepared by combining said multipotent neural stem cell progeny with fresh substantially serum-free culture medium containing one or more predetermined growth factors which induces multipotent neural stem cell proliferation to proliferate said daughter multipotent neural stem cells to produce more progeny which include more daughter multipotent neural stem cells.

22. The method of claim 1 wherein said mammalian neural tissue is obtained from a juvenile or adult.

23. The method of claim 1 wherein said mammalian neural tissue is obtained from a human.

24. A method of producing genetically modified neural cells comprising:

(a) obtaining cells derived from juvenile or adult mammalian neural tissue containing at least one multipotent neural stem cell capable of producing progeny that are capable of differentiating into neurons, astrocytes, and oligodendrocytes;

(b) preparing a culture medium containing one or more predetermined growth factors capable of inducing multipotent neural stem cell proliferation; and

(c) preparing a cell culture by combining the cells obtained in (a) with the culture medium prepared in (b) to induce proliferation of said multipotent neural stem cell to produce multipotent neural stem cell progeny which includes daughter multipotent neural stem cells and introducing exogenous DNA that does not contain oncogenes to genetically modify said multipotent neural stem cell progeny.

25. A method of producing genetically modified neural cells comprising:

(a) obtaining cells derived from human neural tissue containing at least one multipotent neural stem cell capable of producing progeny that are capable of differentiating into neurons, astrocytes, and oligodendrocytes;

(b) preparing a culture medium containing one or more growth factors capable of inducing multipotent neural stem cell proliferation; and

(c) preparing a cell culture by combining the cells obtained in (a) with the culture medium prepared in (b) to induce proliferation of said multipotent neural stem cell to produce multipotent neural stem cell progeny which includes daughter multipotent neural stem cells

and introducing exogenous DNA that does not contain oncogenes to genetically modify said multipotent neural stem cell progeny.

26. The method of claim 1 further comprising:

(d) subjecting said genetically modified multipotent neural stem cell progeny to culture conditions that induce neural cell differentiation to produce a cell culture comprising genetically modified, differentiated neural cells selected from the group consisting of astrocytes, neurons, oligodendrocytes, and combinations thereof.

27. The method of claim 26 wherein in (d) the genetically modified multipotent neural stem cell progeny are differentiated in a culture medium containing serum.

28. The method of claim 26 wherein in (d) the genetically modified multipotent neural stem cell progeny are differentiated on a fixed substrate to which said multipotent neural stem cell progeny can adhere.

29. The method of claim 26 wherein in (d) the genetically modified multipotent neural stem cell progeny are differentiated in a culture medium containing a growth factor that influences differentiation.

30. A method of producing genetically modified differentiated neural cells comprising:

(a) obtaining cells derived from mammalian neural tissue containing at least one multipotent neural stem cell capable of producing progeny that are capable of differentiating into neurons, astrocytes, and oligodendrocytes, wherein said obtained cells have not been cultured in a serum-containing medium;

(b) preparing a substantially serum-free culture medium containing one or more predetermined growth factors capable of inducing multipotent neural stem cell proliferation,

(c) preparing a cell culture by combining the cells obtained in (a) with the culture medium prepared in (b) to induce proliferation of said multipotent neural stem cell to produce proliferating multipotent neural stem cells and multipotent neural stem cell progeny which includes daughter multipotent neural stem cells, and

(d) subjecting the cells proliferated in (c) to culture conditions that induce neural cell differentiation to produce differentiating and differentiated neural cells, and introducing exogenous DNA that does not contain oncogenes to genetically modify said differentiated and/or differentiating neural cells to produce genetically modified differentiated neural stem cell progeny.

31. A composition comprising genetically modified multipotent neural stem cell progeny prepared by the method of claim 1.

32. A composition according to claim 31 wherein said genetically modified multipotent neural stem cell progeny are genetically modified to express a biologically active substance selected from the group consisting of growth factor products, growth factor receptors, neurotransmitters, neurotransmitter receptors, neuropeptides, and neurotransmitter-synthesizing genes.

33. A composition comprising genetically modified differentiated multipotent neural stem cell progeny prepared by the method of claim 26.

34. A composition comprising genetically modified differentiated multipotent neural stem cell progeny prepared by the method of claim 30.

35. A composition comprising genetically modified differentiated neural cells produced by the method of claim 30.

36. A composition according to claim 35 wherein said genetically modified differentiated neural cells are genetically modified to express a biologically active substance selected from the group consisting of growth factor products, growth factor receptors, neurotransmitters, neurotransmitter receptors, neuropeptides, and neurotransmitter-synthesizing genes.

37. A composition comprising a population of non-primary neural cells which are derived from a primary cell culture, said population of non-primary neural cells having a greater percentage of multipotent neural stem cells compared to that of said primary culture, wherein a single multipotent neural stem cell is capable of producing progeny that are capable of differentiating into neurons, and glia, including astrocytes, and wherein said multipotent neural stem cells are genetically modified and do not contain exogenous oncogenes.

38. The composition of claim 37 wherein said percentage of multipotent neural stem cells of said population of non-primary neural cells is at least ten fold higher than that of said primary cell culture.

39. The composition of claim 37 wherein said multipotent neural stem cells are derived from human neural tissue.

40. The composition of claim 37 wherein said multipotent neural stem cells are derived from juvenile or adult neural tissue.

* * * * *

Purification and characterization of chlorotoxin, a chloride channel ligand from the venom of the scorpion

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DeBin, John A., John E. Maggio, and Gary R. Strichartz. Purification and characterization of chlorotoxin, a chloride channel ligand from the venom of the scorpion. *Am. J. Physiol.* 264 (*Cell Physiol.* 33): C361-C369, 1993.—We have previously demonstrated that the venom of the scorpion *Leiurus quinquestriatus* blocks small-conductance Cl^- channels, derived from epithelial cells, when applied to the cytoplasmic surface. We have now purified to near homogeneity, and characterized, the component responsible for this blocking activity. It is a small basic peptide of 4,070 Da. The primary amino acid structure shows considerable homology to a class of previously described putative short insectotoxins. A brief characterization of the kinetics of Cl^- channel block as well as a demonstration of toxicity to arthropods is also presented.

anion channel; scorpion toxin; scorpion venom; insectotoxin; *Leiurus quinquestriatus quinquestriatus*

SCORPIONS COMPRISE an order (Scorpionidae) within the arthropod phylum so successful in nature that their basic anatomy and physiology have not changed for hundreds of thousands of millennia. Because scorpions prey primarily on other arthropods, they have developed the ability to produce and deliver small quantities of very potent toxins to their prey. These basic peptide toxins rapidly paralyze the scorpion's victim, primarily by disrupting normal ion channel function in the nervous system. From the Buthinae family, α- and β-neurotoxins, which alter fast Na^+ channel gating (8, 35, 41) and charybdotoxin (7) and leiurotoxin (6), blockers of Ca^{2+}-activated K^+ channels, have been described. The α- and β-toxins are a large class of toxins, 60-70 amino acids in length, that are generally effective against vertebrates. A structurally homologous group of peptides is known to be effective against either insects or crustaceans. The selectivity of these "long" neurotoxins is, however, not absolute. Some of the mammalian toxins also show considerable toxicity to arthropods (35; cf. Table 4 in Ref. 41), and many of the insect toxins are effective against crustaceans (12, 17). A series of short, 30-36 amino acid, putative insectotoxins (see DISCUSSION) from the *Buthus* and *Androctonus* genera have also been described (35, 40).

Previously we reported that crude venom extracted from the scorpion *Leiurus quinquestriatus quinquestriatus* inhibits reconstituted small-conductance Cl^- channnels isolated from rat epithelia and embryonic rat brain (10, 11). In this paper we describe the purification and characterization of the active component from crude *Leiurus* venom. We now know that this activity is due to a 4.1-kDa basic peptide with considerable primary sequence homology to the small insectotoxins. In recognition of the affinity of this previously undescribed peptide for Cl^--selective ion channels, we have named it "chlorotoxin."

MATERIALS AND METHODS

Rat colonic epithelial chloride channel reconstitution. Rat colonic enterocyte plasma membranes were prepared using the method described by Reinhardt et al. (34) with the following modifications. Male Sprague-Dawley rats, 250-300 g, six to eight at a time were used without exposure to dexamethasone. After decapitation, colons were dissected, washed with cold Ringer solution, everted onto a glass rod, and incubated at 37°C in a solution consisting of (in mM) 30 EDTA, 107 NaCl, 25 $NaHCO_3$, 4.5 KCl, 1.8 Na_2HPO_4, 0.2 NaH_2PO_4, and 12 glucose, pH 7.4. No attempt was made to remove the muscularis or submucosal layers. Over the next hour, the solution bathing the everted colons was bubbled with 5% CO_2, and each colon was gently scraped with a metal spatula every 10 min. After 60 min, the entire suspension of epithelial cells and sheets of cells were centrifuged at 600 *g* for 15 min at 4-6°C, and the pellet was resuspended in an equal volume of 8.5% sucrose (wt/vol), 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 5 mM imidazole, pH 7.4.

The remainder of the protocol was carried out at 4-6°C and is as described in Ref. 34. In brief, this procedure entails washing the pellet two additional times in the 8.5% sucrose solution described above. After the third wash, the pellet was resuspended in approximately twice its volume of 8.5% sucrose solution and homogenized (20 strokes) with a tight-fitting (A) Dounce homogenizer. After centrifugation at 1,200 *g* for 15 min, the supernatent was saved and the pellet resuspended and rehomogenized as before. After four to five cycles of homogenization, the pooled supernatant fraction was centrifuged at 22,000 *g* for 75 min. Most of the pellet (≈3 ml total excluding the dense, yellow-white button present at the very bottom of the centrifuge tube) was then recovered and resuspended in 6 ml of 8.5% sucrose solution with the aid of a loose-fitting (B) Dounce homogenizer. This suspension of membranous particles was then layered on top of a discontinuous sucrose gradient consisting of 40% (wt/vol) sucrose, 1 mM EGTA, and 5 mM imidazole, pH 7.4, and 20% sucrose, 1 mM EGTA, and 5 mM imidazole, pH 7.4. After centrifugation at 130,000 *g* for 2 h, the material at the 20/40% interface was collected, and the entire volume (≈5 ml) was diluted to 30 ml with the 8.5% sucrose solution. After a final centrifugation at 27,000 *g* for 60 min, a small pellet (≈400 μl) was collected and resuspended in an equal volume (400 μl) of the 8.5% sucrose, 1 mM EGTA, and 5 mM imidazole, pH 7.4, solution. Subsequent to aliquoting, this suspension of membranes was stored at −80°C and used for up to 6 mo after the date of preparation.

Chloride channels were reconstituted from the rat enterocyte preparation into planar phospholipid bilayers using well-established methods (31). Lipid solutions were prepared each day from chloroform stock solutions. After removing the chloroform with a stream of N_2, 40 μg of 1-palmitoyl-2-oleoyl phosphatidylethanolamine and 10 μg of 1-palmitoyl-2-oleoyl phosphatidylcholine (nos. 850757 and 850457; Avanti Polar Lipids, Alabaster, AL) were redissolved in 20 μl *n*-decane. The sealed end of a glass capillary tube was used to apply a small amount of this solution across a 250-μm-diameter hole drilled through a 2-mm-thick polystyrene partition separating two (2.5 and 4 ml) chambers. The solution used in each chamber at the

[illegible] solution consisted of 200 mM NaCl, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and 0.2 mM EGTA (pH 7.4). A NaCl gradient (200 mM cis bath, 50 mM trans bath), used to enhance channel incorporation, was collapsed before the beginning of an experiment by adding concentrated NaCl to the trans bath. Chloride channels were usually seen within 30 min of pipetting 5 µl of a suspension of plasma membrane [illegible] protein [illegible] near the bilayer on the cis-side. All [illegible] experiments were conducted at ambient temperature [illegible].

Single chloride channel recording. Cis- and trans-baths were [illegible] M KCl [illegible] salt bridges and Ag-AgCl [illegible] Clampex, CT [illegible] [illegible] digital data for storage on a videocassette recorder tape using a Sony PCM-501 [illegible] The data were subsequently filtered at [illegible] model [illegible] in the [illegible] and then analyzed using a PC's [illegible] computer and Axon Instruments TL-1 interface in conjunction with the single-channel programs found in PClamp software (Axon Instruments, Foster City, CA). Junction potentials measured at the end of each experiment were found to be <5 mV in all cases, and thus were ignored.

Ion channels were identified as being chloride selective by the direction of current flux at 0 mV applied potential in the presence of a NaCl gradient. Enterocyte Cl^- channels also displayed properties similar to those described by other investigators for reconstituted channels from the rat colon (4, 34): curvilinear current-voltage relationships with ≈50-pS conductance at +20 mV and ≈32 pS at −20 mV in symmetrical 200 mM NaCl, spontaneous gating with frequent rapid closures, and a reduction in current amplitude to one-half control by 5 µM 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS) applied "extracellularly." Because the Cl^- channels were found to incorporate into bilayers in both of two possible orientations (cytoplasmic surface facing either the *cis*- or *trans*-bath), channel sidedness was determined from the current rectification properties of the individual channels, based on the assumption that the reconstituted enterocyte Cl^- channels are outwardly rectifying, like those described in intact cells using patch-clamp electrodes (14, 25). All voltages are, therefore, reported as the true membrane potential according to established conventions (i.e., outward rectification of an anion current would imply a greater flux of anions into a cell than in the reverse direction in response to applied potentials of equal magnitude but opposite polarity).

A current amplitude exactly halfway between the maximum steady-state open channel and zero-current levels was chosen as the threshold for the detection of an opening/unblocking or closing/blocking transition. Because at steady state the enterocyte Cl^- channels spontaneously close for brief periods and reopen (cf. controls, Figs. 2 and 3), channel block by ligand molecules must be distinguished from random channel gating. Because most of the spontaneous closures in the absence of ligand are of short duration (i.e., <500 ms), a cut-off time (t_c) of 1.5 s was chosen such that only events lasting longer were considered channel blocks. On the basis of the frequency of spontaneous closures of >1.5-s duration in control records, the potential maximum "contamination" of true channel blocks by spontaneous closures was determined to be <10% in all cases. In other words, for a given time interval, the number of zero-current events (i.e., closures/blocks) lasting longer than 1.5 s was at least nine times greater in the presence of ligand than in its absence. Moreover, the use of a t_c of 0.5 or 1.0 s resulted in less than a 10% change in the values obtained for mean open/unblocked (t_o) or mean blocked (t_b) times.

The use of a t_c for the detection of channel blocking events will, however, cause the exclusion of a number of brief channel blocks and thus result in artificially high determinations for t_b and t_o. The correction described by Moczydlowski et al. (32) was therefore used to arrive at "true" t_b and t_o values.

With the assumption that the binding of one ligand molecule to a Cl^- channel in the open state (see RESULTS) is sufficient to induce a channel block, and that each binding results in a channel block, the dissociation constant (K_d) and the rate constants for the binding (k_{+1}) and unbinding (k_{-1}) reactions can be obtained from t_b and t_o according to the following scheme

$$C_c \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} C_o \overset{L}{\underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}}} CL$$

where L represents the ligand molecule and C_c and C_o the Cl^- channel in the closed and open states, respectively. The dissociation rate constant k_{-1} is simply equal to $(t_b)^{-1}$, while the bimolecular association rate constant k_{+1} is equal to $[\beta - (t_o)^{-1}]/[L]$, and K_d equals k_{-1}/k_{+1}. The intrinsic rate of channel closure at steady state (β) is equivalent to the reciprocal mean open time determined in the absence of ligand. For the case in which the ligand concentration is known only in terms of milligrams per milliliter, the inhibitory constant (K_I) (or $K_{0.5}$) can be defined as $\{L/[t_b/(t_b + t_o)]\} - L$. Values for blocking parameters and equilibrium constants are given as means ± SD.

The mass of purified peptide present in stock solutions was determined by integration of the chromatograms derived from the digests performed for the analysis of amino acid content as described below. We also performed Lowry protein assays on these same stock solutions to establish the ratio between protein concentration as determined by Lowry assay and true protein content as determined from the amino acid analyses. This ratio was determined to be 1.81, indicating that the Lowry method overestimates by nearly twofold the concentration of this particular peptide. In some instances, Lowry assays were performed to determine the protein concentration of various peptide fractions. In these instances the ratio determined above was used to correct the values obtained.

Crude venom source and processing. All venom for the experiments described in this paper was that of *Leiurus quinquestriatus quinquestriatus* obtained from Latoxan (Rosans, France). All venom was dispersed in deionized H_2O, at 20 mg dry venom/ml, using a Potter-Elvehjem tissue grinder. After pelleting of the ubiquitous tenacious mucous in a swinging bucket rotor at 10,000 *g* for 30 min, the upper, relatively mucous-free, solution was recovered with a Pasteur pipette. This was in turn filtered using an Amicon Micropartition System (Beverly, MA) with 10-kDa cut-off filters at 1,690 *g* in a fixed angle rotor. The filtrate was collected, vacuum centrifuged, and reconstituted in 10 mM trifluoroacetic acid (TFA) at a concentration of ~20 mg/ml and subsequently loaded, 1 ml at a time, onto a Waters C_{18} Sep-Pak cartridge. The fraction containing the active component was eluted with 25% acetonitrile in 10 mM TFA, vacuum centrifuged to dryness, and reconstituted in 10 mM TFA in preparation for reverse-phase high-pressure liquid chromatography.

Reverse-phase high-pressure liquid chromatography (RP-HPLC). All RP-HPLC was performed using linear gradients of acetonitrile in either of two ion pair reagents as indicated in text: 10 mM TFA or 5 mM heptafluorobutyric acid (HFBA). We employed a Vydac TP-54 C_{18} column (Nest Group, Southborough, MA) for all work. Flow rate was a constant 1 ml/min in all cases, and absorbance was monitored at 214 nm. For preparative work, loads of 100 µg processed venom in 0.3 ml of 10 mM TFA were used. Loads for analytical work were as indicated in the legend to Fig. 1.

Ion-exchange HPLC. Peptides purified by RP-HPLC were repurified by ion-exchange HPLC using a polysulfoethyl

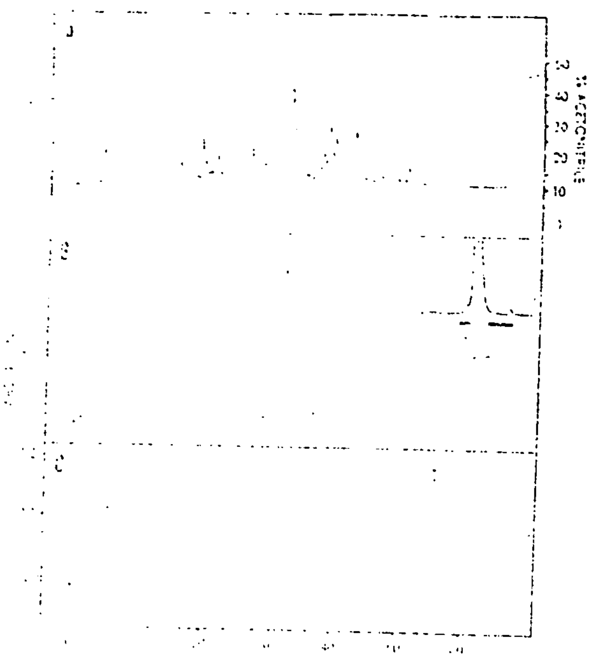

FIG. 1. Reverse-phase high-pressure liquid chromatography (RP-HPLC) chromatogram of processed *Leiurus quinquestriatus quinquestriatus* venom (*A*) and the purified ligand using 2 different ion pair reagents (*B* and *C*) are shown. A Vydac TP-54 C_{18} column was used for all separations. The same linear gradient of acetonitrile was used in each case, and this is shown in *A* only with percent acetonitrile indicated on *right axis*. Chromatograms have been drawn so that sample injection points (▲) are aligned vertically. Buffer flow rate was 1 ml/min in each case, and absorbance was continuously monitored at 214 nm. Note change in absorbance scales on *left axis*. In each panel, peak containing active component is indicated by an asterisk. *A*: crude scorpion venom was processed by filtration through 10-kDa cut-off filters as described in MATERIALS AND METHODS. Twenty micrograms of this processed venom were reconstituted in 0.3 ml of 10 mM trifluoroacetic acid (TFA) and loaded onto the column and eluted with a linear gradient of acetonitrile in 10 mM TFA. When the lag between elution profile and gradient is taken into account, active component is seen to emerge between 24 and 26% acetonitrile. *B*: 3 μg purified chlorotoxin were reconstituted in 0.3 ml of 10 mM TFA, loaded, and eluted with an acetonitrile gradient in 10 mM TFA as in *A*. *Inset*: enlargement of base of main peak. Areas collected as α- and γ-peak material are indicated by thick dark lines underneath chromatogram. *C*: 3 μg chlorotoxin were reconstituted in 0.3 ml of 5 mM heptafluorobutyric acid (HFBA) and eluted with 5 mM HFBA in acetonitrile. Injection artifact, small in *A* and *B*, encompasses nearly the first 20 min of this chromatogram. Small peak emerging at 40 min (○) was apparent also in blank (control) chromatogram.

aspartamide cation-exchange column (no. P1950-204; Nest Group). A linear gradient of NaCl (0–1.35 M) in 10 mM phosphate and 10% acetonitrile at constant pH 4.0 was used as indicated in Fig. 6. Flow rate was a constant 1 ml/min throughout, and the absorbance of the emerging peptides was continuously monitored at 280 nm.

Amino acid composition and primary sequence. Three separate amino acid analyses were performed. The first two analyses were performed [illegible] 10 μg of purified peptide in 6 M [illegible] amino acid composition of the hydrolysate was then determined following phenylisothiocyanate derivatization and chromatography using an Applied Biosystems 420A derivatizer and 130A separation system. A third analysis was performed by digesting the peptide in 6 N HCl for 22 h at 110°C under N_2. The amino acid composition was determined using a Beckman 6300 analysis system following conjugation with ninhydrin.

In preparation for sequencing, 40 μg of purified peptide were reduced and alkylated by first dissolving in 50 μl of 8 M urea, 0.4 M NH_4CO_3, pH 8.0. Dithiothreitol was added to a final concentration of 4 mM, and the solution was heated to 50°C for 30 min in a closed 1.5-ml polypropylene tube. Iodoacetamide was then added to a final concentration of 10 mM. After 15 min, the solution was diluted fourfold in 10 mM TFA and loaded onto a Waters C_{18} Sep-Pak. The reduced alkylated peptide was eluted with 100% acetonitrile, vacuum centrifuged to near dryness, and submitted for sequencing.

Sequencing was performed at the Harvard Microchemistry Facility, Cambridge, MA. In brief, an aliquot of alkylated peptide was applied to a polybrene precycled glass fiber filter and placed into the reaction chamber of an Applied Biosystems 477A Protein Sequencer equipped with an on-line phenylthiohydantoin (PTH) analyzer. Released PTH amino acids were subsequently identified by direct observation of the chromatogram. The sequence cycle, NORMAL-1, was modified using the manufacturer's recommendations for faster cycle time (37 min) by decreasing dry down times and increasing reaction cartridge temperatures to 50°C during coupling.

Arthropod toxicity experiments. All animals were obtained from Connecticut Valley Biological Supply (Southampton, MA). Crayfish (*Procambis clarkii*) weighing 4–6 g were injected with purified chlorotoxin (1.23–2.23 μg/g body wt) in 10 μl H_2O. Larger crayfish (9–10 g) received smaller doses of chlorotoxin (0.5 μg/g) as described in greater detail in RESULTS. In all cases the injections were made through the ventral surface of the thorax in the region of the subesophageal ganglion, using a 25-gauge needle on a 1-ml syringe. All crayfish served as their own controls and were first injected with 10 μl H_2O to which no response was observed for up to 3 min. Large (≈3 cm length) American cockroaches (*Periplaneta americana*) were similarly injected through the ventral surface at the thoracoabdominal junction using a 25-gauge needle. An equivalent volume of H_2O injected into control subjects was without effect as described in RESULTS.

The injection of small volumes of toxin was accomplished by first placing the 10-μl aliquot in a 1.5-ml test tube. The plunger of the syringe was then pulled back to draw roughly 50 μl of air into the syringe, and then the 10-μl aliquot of toxin or vehicle (H_2O) was drawn up. By injecting the solution first followed by the small bolus of air, we ensured the delivery of the entire amount of toxin.

RESULTS

Purification of the active component from Leiurus venom. Processed crude *Leiurus quinquestriatus quinquestriatus* venom was loaded onto a C_{18} RP-HPLC column and eluted with a linear gradient of acetonitrile in 10 mM TFA. The resulting chromatogram is shown in Fig. 1*A*. Individual peaks were collected by hand, taken to dryness, and reconstituted in 0.5 ml of 10 mM TFA. As an initial screen, small aliquots from five to six individual peaks were pooled and assayed for activity using reconstituted rat colonic enterocyte Cl^- channels, as described in MATERIALS AND METHODS. Once activity was localized to a general area of the HPLC chromatogram, the individual peak containing the active component (asterisk in Fig. 1) was identified using the same reconstituted Cl^- channel assay. A subsequent, large preparative purification was then accomplished in several separate runs, using the same HPLC conditions. The peaks containing the active component, easily identifiable by their size and

fractions on the elution profile, were collected, pooled, and reconstituted in 200–500 μl of TFA buffer. This partially purified component was subjected to a second round of chromatography under identical conditions in which only the center of the large peak, from several runs was collected, pooled, and reconstituted. This material was used for all blocking and arthropod toxicity experiments.

Effects of crude venom and purified ligand on reconstituted Cl⁻ channels. When crude *Leiurus* venom is applied to the cytoplasmic surface of a single reconstituted rat colonic enterocyte Cl⁻ channel, a potent inhibition results as seen in Fig. 2. This effect is seen only with application to the intracellular surface; no block is seen with application to the extracellular channel face. In the control record, open channel probability is high (P_o > 90%) with only occasional brief, reversible transitions from the open state to the closed or zero-current level. After exposure to 0.2 mg venom/ml, the Cl⁻ channel

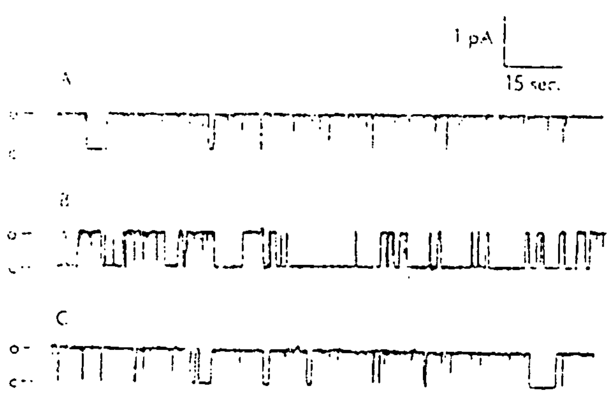

Fig. 2. Effects of crude venom on a reconstituted Cl⁻ channel are shown by 3 current traces from the same single rat enterocyte Cl− channel in a bilayer held at −20 mV. Methods of reconstitution were as described in MATERIALS AND METHODS. All records were filtered at 50-Hz low pass. o, Open/unblocked state; c, closed/blocked state. Scale bars represent 1 pA and 15 s. *A*: control situation before addition of venom [open channel probability (P_o) = 0.91]. *B*: immediately (<30 s) subsequent to addition of venom to 0.2 mg/ml final concentration in the "intracellular" bath (P_o = 0.31). *C*: washout: immediately after perfusion of intracellular bath with 4 vol of fresh buffer (P_o = 0.68).

spends more time in the closed/blocked state than in the open state. There is no reduction in open-state current amplitude, indicative of a rapid open channel blocker, and there are no detectable subconductance states induced by the venom.

Previously we reported that the blocking activity of crude venom on single enterocyte Cl⁻ channels was characterized by a dose-dependent shortening of open time accompanied by no change in t_0 (11). These results, which are paralleled by the actions of purified ligand, are consistent with the selective binding of ligand to open channels. The inhibition by crude venom described here results from discrete blocking events lasting 5.23 ± 0.59 (SD) s at −20 mV (n = 4), on average, corresponding to a k_{-1} of 0.19 ± 0.02 s^{-1} (Table 1). From the values for mean open and closed times, a K_I for Cl⁻ channel inhibition of 0.11 mg venom dry wt/ml is obtained at −20 mV.

When 2.42 μg/ml of the purified ligand is applied to the cytoplasmic surface of a reconstituted enterocyte Cl⁻ channel, a reduction in P_o occurs that is similar to that induced by the crude venom (Fig. 3). Assuming that the binding of one ligand molecule is sufficient to induce channel block, we can calculate both k_{-1} and k_{+1} from mean block and mean open times, as described in the MATERIALS AND METHODS. Furthermore, from rate constants for the blocking reaction we can determine the true K_d for the ligand-channel interaction. From control records the intrinsic channel closing rate, β, was determined to be 0.016 s^{-1} and 0.015 s^{-1} at −20 and +20 mV, respectively.

Given a molecular mass of 4,070 Da from the amino acid sequence, a K_d of 1.15 μM (4.68 μg/ml) at −20 mV was calculated. When compared with the K_I for channel block by venom, this indicates that the active ligand is ~4.3% of crude venom dry weight. Given the affinity of this ligand for Cl⁻-selective ion channels, and its toxicity to arthropods (see below), we have elected to name it chlorotoxin.

A comparison of the blocking kinetics of an enterocyte channel at +20 mV to those at - 20 mV (Fig. 3) indicates that chlorotoxin binding is voltage dependent.

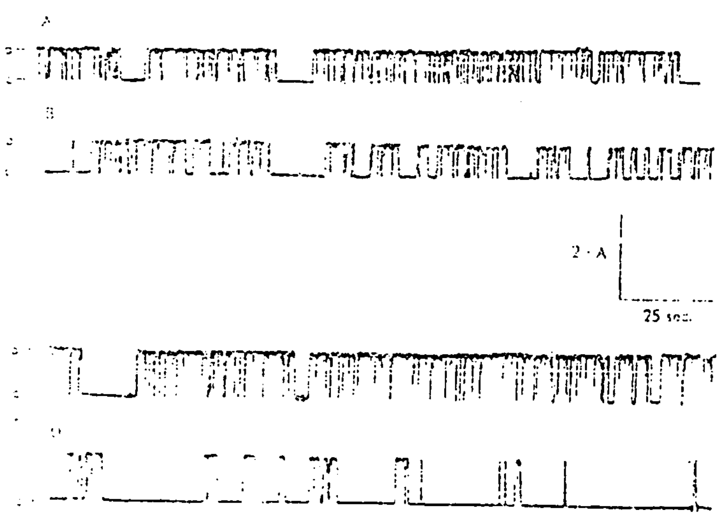

Fig. 3. Chlorotoxin block of a reconstituted Cl⁻ channel is shown. Current traces are from same reconstituted rat enterocyte Cl− channel before and after addition of purified chlorotoxin. As in Fig. 2, all records have been filtered at 50-Hz low pass. o, Open/unblocked current amplitude; c, closed/blocked state. Both control traces (*A*, −20 mV; *C*, +20 mV) were obtained before addition of any ligand. Scale bars represent 2 pA and 25 s. *A*: control at −20 mV; before addition of chlorotoxin (P_o = 0.80). Note that compared with Fig. 2*A*, there is considerably more intrinsic gating at −20 mV demonstrated by channel shown here. Such variations in channel behavior were found to be common. *B*: −20 mV; 7 min after addition of 594 nM chlorotoxin to the "intracellular" bath (P_o = 0.60). *C*: control at +20 mV; before adding ligand (P_o = 0.94). *D*: +20 mV; immediately after exposure to 594 nM chlorotoxin (P_o = 0.18).

Table 1. *Chloride channel blocking data for Leiurus venom and purified chlorotoxin*

	Membrane potential, mV	t_b, s	k_{-1}, s^{-1}	k_{+1}, $s^{-1} \cdot M^{-1}$	K_I, K_d
Crude venom	−20	5.23±0.59	0.19±0.02		0.11±0.02 mg/ml
Chlorotoxin	−20	3.63±1.06	0.29±0.09	$(2.80\pm0.63)\times10^5$	1.15±0.61 μM (4.68 μg/ml)
Chlorotoxin	+20	11.85±2.76	0.09±0.02	$(5.07\pm0.84)\times10^5$	180.6±70.9 nM (0.74 μg/ml)

Values are means ± SE. Data represent a summary of kinetics of single Cl^- channel block by crude *Leiurus* venom and purified chlorotoxin. [illegible] channel recording conditions are described in MATERIALS AND METHODS, where a description of the various parameters is also given. All [illegible] single Cl^- channels held at either −20 or +20 mV membrane potential as indicated. Four individual Cl^- channels were analyzed [illegible] the rate constants for block by crude venom or by chlorotoxin +20 mV, and 5 channels were analyzed for block by chlorotoxin at 20 mV [illegible] analysis. Two different concentrations of chlorotoxin were used, either 594 nM or 1.5 μM. Crude venom was used at 0.2 mg/ml [illegible] The K_I for crude venom is given on the basis of the dry weight of whole venom used before extraction of mucous.

This voltage dependence seems to result primarily from a slowing of the dissociation rate, as evidenced by the approximate threefold decrease in k_{-1} during depolarization (0.29 ± 0.09 s^{-1} at −20 mV, n = 5; compared with 0.09 ± 0.02 s^{-1} at +20 mV, n = 4) in addition to a nearly twofold enhancement of the rate of binding (k_{+1} = 2.80 ± 0.63 × 10^5 $M^{-1} \cdot s^{-1}$ at −20 mV, n = 5; compared with 5.07 ± 0.84 × 10^5 $M^{-1} \cdot s^{-1}$ at +20 mV, n = 4). From the rates of channel block and unblock at +20 mV, a K_d of 180.6 ± 70.9 nM (0.74 μg/ml) can be determined. Thus the ligand's affinity increases more than fivefold between −20 and +20 mV membrane potential.

Open time (unblocked) and closed time (blocked) histograms for the data obtained at −20 mV are shown in Fig. 4. Both histograms could be fit by single exponential functions. The closed duration time constant (τ_b) was 2.84 s, which is close to the value for t_b (3.63 s) given in Table 1. The open duration time constant (τ_o) taken from the histogram was 3.62 s, which is close to the value of 4.92 ± 0.94 s determined for mean t_o for the three channels blocked by 594 nM chlorotoxin. Due to the long duration of the blocking events at +20 mV, we were unable to collect enough events to construct meaningful histograms at this potential.

Amino acid composition and primary structure. An analysis of the amino acid composition of the purified material was performed in triplicate using two different methods. The results (Table 2) indicate that chlorotoxin is a basic peptide of 30–35 amino acids. This conclusion is supported by Sephadex G-50 chromatography in which the active component eluted between markers microperoxidase (1,899 Da) and aprotinin (6,400 Da).

The primary amino acid sequence of the S-alkylated peptide was determined, and the results are shown in Fig. 5. The total yield of amino acid for the first cycle was 65% of the total mass loaded, indicating that the sequenced material was indeed the major component rather than a contaminating peptide.

The primary structure of chlorotoxin is shown in Fig. 5 along with the sequence of three other putative short insectotoxins. From the amino acid sequence a molecular mass of 4,070 Da can be determined for chlorotoxin (4,062 Da assuming four disulfide bonds).

Effects of purified chlorotoxin on arthropods. The effects of the purified ligand on crayfish were assessed as described in MATERIALS AND METHODS. Chlorotoxin at 1.23–2.23 μg/g body wt produced a loss of motor control begining at ≈20 s after injection, which progressed to a rigid paralysis of the walking and pincer legs that was complete within an additional ≈40 s. Within ≈90 s of injection, the tail musculature was immobilized. A total of four crayfish were injected this way with similar results from which no recovery was noted for 6 h, at which time the crayfish were destroyed. In four additional crayfish, chlorotoxin, at 0.5 μg/g, induced the same progressive paralysis with a slower onset, beginning at roughly 1 min postinjection. The paralysis was complete within 3–4

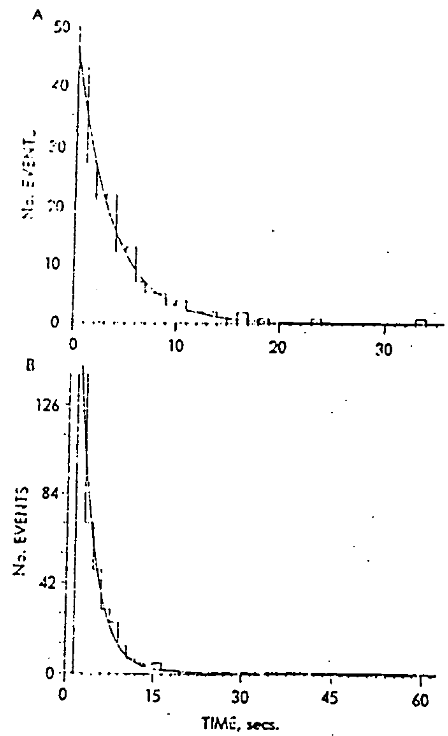

Fig. 4. Open and closed time histograms for chlorotoxin block of anion channels at −20 mV are shown. Histograms shown are probability density histograms generated by curve-fitting programs in PClamp software for the block of rat colonic enterocyte Cl^- channels by purified chlorotoxin. Membrane potential was −20 mV in each case, and a cut-off time of 1.5 s was used in the detection of channel closings/blocks, as described in MATERIALS AND METHODS. *A*: open time histogram; open/unblocked distribution for data from three channels blocked by 594 nM chlorotoxin. There were 174 total events fit from 0–35 s, yielding a τ_o of 3.62 s. *B*: closed time histogram, closed/blocked duration histogram for data from the 3 channels in *A*, plus 2 additional channels blocked by 1.55 μM chlorotoxin. There were 362 total events, and the exponential function was fit only from 1.5 to 60 s (because of the use of a cut-off time of 1.5 s) to yield a τ_b of 2.84 s.

Table 2. *Amino acid composition of chlorotoxin and other select Buthinae toxins*

Amino Acid	Mean Number of Amino Acids From Chlorotoxin Digests	Number of Amino Acids From Chlorotoxin Sequence	Mean Number of Amino Acids Reported for a Selection of Buthinae Toxins
Asx	[illegible]	[illegible]	9.2±0.8
[illegible]	[illegible]	[illegible]	2.9±1.6
Ser	[illegible]	[illegible]	3.1±1.3
Glx	[illegible]	[illegible]	6.2±0.9
His	1.00 [illegible]	1	0.9±0.5
Arg	[illegible]	[illegible]	2.5±1.0
Thr	1.82 (2)	2	2.0±1.0
Ala	1.01 (1)	1	3.7±1.6
Pro	1.91 (2)	2	3.1±1.2
Tyr	[illegible]	1	5.2±1.5
Val	[illegible]	0	3.5±1.5
Met	1.75 (2)	[illegible]	0
Ile	0.11 (0)	0	2.5±1.0
Leu	1.02 (1)	1	2.8±0.9
Phe	1.10 (1)	1	1.0±0.5
Lys	2.77 (3)	3	5.0±1.2

Values in *middle column* were obtained from primary sequence of chlorotoxin, and numbers in the *right column* are averages (±SD) of 19 different Buthinae toxins taken from Lazarovici and Zlotkin (29). Mean number of each amino acid was the average of 3 determinations obtained from digests of purified chlorotoxin as described in MATERIALS AND METHODS. For each individual determination, number of amino acids per molecule of chlorotoxin was calculated by dividing picomoles of each individual amino acid, recovered from digest and subsequent separation, by the average number of picomoles obtained for His, Ala, Leu, and Phe residues. Number in parentheses is the mean of the 3 determinations rounded to nearest whole number.

min. After 2 h, all crayfish had recovered from the effects of 0.5 $\mu g/g$ chlorotoxin.

The injections of insects produced results similar to those observed in crayfish. Three large American cockroaches were each injected with 4.5 μg chlorotoxin. Within 30–60 s, each was immobilized with its legs contracted over its abdomen, a condition in which it remained for at least the next 2 h. After 20 h, two of the roaches had fully recovered, while the third showed a partial recovery of leg mobility. Three cockroaches injected with H_2O remained fully active throughout the ensuing 24 h.

Confirmation of chlorotoxin activity: repurification of chlorotoxin. The chlorotoxin used for all experiments described above was obtained from a two-step RP-HPLC purification protocol. This raises the possibility that the bioactivity reported for chlorotoxin may in fact be due to a contaminating peptide, present in small quantities, which copurifies with chlorotoxin. To exclude this possibility, purified chlorotoxin was repurified under different conditions to determine if it retains the bioactivity described above.

A small amount (≈500 μg) of the material from the second round of reverse-phase chromatography was subjected to yet a third round of purification using the same C_{18} column and a linear gradient of acetonitrile in 10 mM TFA (cf. Fig. 1*B*). This time not only was the central peak collected, but the small leading and trailing peaks (denoted by α and γ, respectively, in Fig. 1*B*, *inset*) were also collected. The fractions collected from several runs were pooled into three groups (α-peak, main peak, and γ-peak material), which were then taken to dryness and reconstituted in the same volume of H_2O. No inhibition was seen when the α- and γ fractions were simultaneously applied to the cytoplasmic surface of a Cl^- channel at twice the concentration that would normally be present along with the amount of chlorotoxin required to reduce P_o by >0.5. Thus these peripherally chromatographed proteins, the major contaminants of chlorotoxin, when used free of the main peak material at concentrations in excess of those that would normally contaminate purified chlorotoxin, appear to have no Cl^- channel blocking activity.

Two crayfish were used to test for toxic effects of the α- and γ-peak material. When a volume of each of the two fractions, equivalent to twice the volume of chlorotoxin solution subsequently to be used to induce paralysis, was injected (both fractions simultaneously) into the crayfish, no effects were noted for 10 min. The subsequent injection of exactly one-half this volume of chlorotoxin (to 0.77 $\mu g/g$) induced the paralysis described above in both crayfish.

Chlorotoxin was also repurified using the C_{18} RP-HPLC column described above except that HFBA was substituted for TFA as the ion pair reagent (Fig. 1*C*). No additional major peaks were observed, a result which confirms the near homogeneity of chlorotoxin. The main peak material (asterisk in Fig. 1*C*) was collected, vacuum centrifuged to dryness, and reconstituted as a concentrated stock solution for use in further testing. When applied to the cytoplasmic surface of a reconstituted Cl^- channel, this material proved to be fully active in inducing the prolonged blocks described for chlorotoxin (cf. Fig. 3). HFBA-purified chlorotoxin was also examined for arthropod toxicity. When injected into two crayfish at ≈1 $\mu g/g$, this material produced a progressive paralysis with

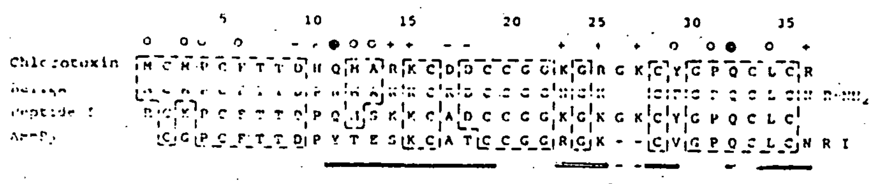

Fig. 5. Comparison of primary amino acid sequence of chlorotoxin with those of 3 small insectotoxins is shown. Primary sequence of chlorotoxin has been aligned with 3 small insectotoxins by matching cysteine residues. NH_2-terminus is on *left* in each case. Amino acids common to all 4 peptides are contained in boxed areas. Above the 4 sequences, positively charged amino acids are denoted by +, negatively charged amino acids by −, polar uncharged amino acids by closed circle, and apolar amino acids by open circle. Below the 4 sequences, extent of hypothesized α- and β-conformations of BeIT$_5$A are indicated by thick lines. See DISCUSSION for further details. Peptide I is from *Buthus sindicus* (18), BeIT$_5$A is from *Buthus eupeus* (1), and AmmP$_2$ is from *Androctonus mauretanicus* (35, 41).

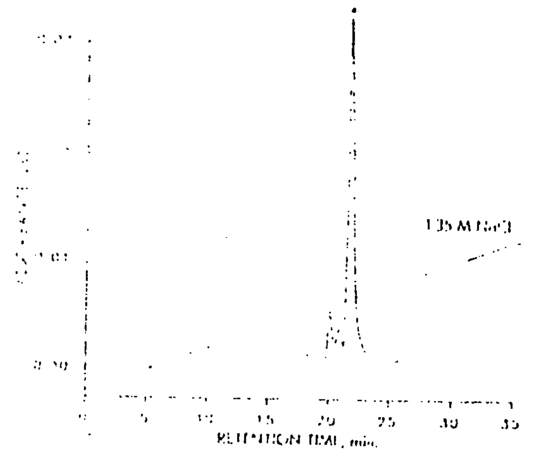

Fig. 6. Ion exchange HPLC of purified chlorotoxin is shown. Chlorotoxin purified via RP-HPLC was repurified using a polysulfoethyl aspartamide ion exchange column, and resulting chromatogram is depicted here. A linear gradient of NaCl (0–1.35 M, in 10 mM phosphate and 10% acetonitrile) was used to elute peptides. Injection of 60 μg purified chlorotoxin took place at time 0, and gradient was begun at 5 min postinjection. Peak containing active component is indicated by an asterisk. Flow rate was constant at 1 ml/min. Note that unlike chromatograms depicted in Fig. 1, absorbance was monitored at 280 nm.

the same time course described above for chlorotoxin used at 1.23 μg/g.

As a final test of the purity of chlorotoxin, 250 μg were rechromatographed using ion-exchange HPLC as described in MATERIALS AND METHODS. The resulting chromatogram is shown in Fig. 6, and again there is a single major peak (denoted by asterisk, representing chlorotoxin) surrounded by a few smaller satellite peaks. The major peak material was collected, reconstituted, and assayed for activity against Cl^- channels and crayfish. The application of this material to the cytoplasmic surface of a Cl^- channel to 2.5 μg/ml final concentration resulted in a reduction of P_o by >50%. Thus chlorotoxin retains its Cl^- channel blocking activity subsequent to repurification using ion-exchange HPLC. At 0.6 μg/g, this material was also effective in paralyzing two crayfish within 90 s of injection.

DISCUSSION

There are several properties of chlorotoxin that are typical of other Buthinae toxins (35, 41). The molecule is small and basic, being highly cationic at physiological pH. Charges are often paired in the primary sequence, as with Asp^9-His^{10}, Arg^{14}-Lys^{15}, and Asp^{17}-Asp^{18}. All the long Buthinae toxins are extensively cross-linked by disulfide bonds, with most containing four such links. The eight cysteine residues of chlorotoxin suggest the presence of four disulfide bonds that would make this molecule especially compact. Despite these similarities, the high methionine and glycine content, and the absence of serine, isoleucine, and valine, make chlorotoxin unique compared with previously described long neurotoxins (Table 2).

The primary structure does show considerable homology with the putative short insectotoxins (18, 35, 40). When cysteine residues are aligned as in Fig. 5, identical amino acids occur at 26 of 36 positions (72%) in chlorotoxin when compared with $BeIT_5A$ (from *Buthus epeus*; Ref. 1). Four additional positions show conservative replacement between chlorotoxin and $BeIT_5A$, resulting in 81% homology. Sequence identity between chlorotoxin and peptide I (from *Buthus sindicus*; Ref. 18) is even greater at 81%. Relative to $BeIT_5A$, major conserved sequences are Met^1-Asp^9, Asp^{18}-Gly^{22}, and Gly^{30}-Cys^{35}. We remark on this homology because $BeIT_5A$ is the only short insectotoxin whose secondary structure has been studied. Using high-resolution two-dimensional nuclear magnetic resonance, Arseniev et al. (1) measured interproton distances in this peptide in acidic aqueous solutions (pH 2.9 and 5.5) and subsequently proposed a three-dimensional conformation. They argued that a right-hand α-helix extended from Asn^{11} to Cys^{19} and that antiparallel β-structures occupied Asn^{23}-Phe^{27} and Gln^{30}-Asn^{34} (19). These regions in $BeIT_5A$ are indicated by the thick lines below the sequences in Fig. 5. Assuming an analogous secondary structure, the highly charged region of chlorotoxin would occur adjacent to and within the α-helical conformation. The remaining three positive residues occur in the next β-structure (Lys^{23}-Tyr^{29}).

Although experimental evidence is lacking, it has been proposed that the short insectotoxins might act on the "nerve system of insects" (33) at the level of the "glutamate receptor of the postsynaptic membrane" (1). However, other investigators believe that the small insectotoxin-like peptides found in several Buthinae venoms are not part of these scorpions' natural secretions but are found only in venom collected by electrical stimulation (36). Manual stimulation, that is stimulation by physically perturbing the scorpion, fails to elicit small insectotoxins. Furthermore, it has been reported that $AmmP_2$, a short insectotoxin, looses its toxicity to arthropods following treatment with antibodies raised against the long insect toxin of *Androctonus australis Hector* (36).

The venom used to isolate chlorotoxin was obtained by electrical stimulation of scorpion poison glands; we have not attempted to isolate chlorotoxin from manually elicited venom. Given that a few of the long insect toxins are effective against arthropods at concentrations as low as 10 ng/g body wt (12, 13), and that we have employed upwards of 500 ng chlorotoxin/g in our tests on live animals, it is conceivable that a contamination could explain the toxicity or Cl^- channel blocking activity of chlorotoxin. However, we consider this unlikely for the following reasons. First, the α- and γ-peak material, the major contaminants of chlorotoxin, when used at concentrations equal to or in excess of those that would normally contaminate chlorotoxin, were completely devoid of activity against either arthropods or reconstituted Cl^- channels. By switching to HFBA as the ion pair reagent for HPLC, no additional peaks were resolved, while the chlorotoxin peak was demonstrated to retain both Cl^- channel blocking activity and toxicity to arthropods. Moreover, after repurification using ion-exchange HPLC, chlorotoxin retains its channel blocking activity and its toxicity. Thus, if either of these two activities of chlorotoxin are in fact due to a contaminating peptide, this peptide would have to copurify with chlorotoxin under

three different HPLC conditions. Finally, in light of our previously published lack of effect of two purified α-toxins, or of charybdotoxin (the most likely types of contaminating peptides derived from *Leiurus* venom) on reconstituted Cl^- channels (11), we consider it unlikely that the Cl^- channel inhibition we see is due to a contaminant.

The Cl^- channels we describe here belong to the large group of neurotransmitter-insensitive "leak" channels found in a wide variety of cell types (2, 5, 9, 20, 23). In recent years these Cl^- channels have become the focus of intense interest because they have been shown to be the site of the defect in the common inherited disease cystic [illegible] The colonic enterocyte Cl^- channels also share several features with both the γ-aminobutyric acid (GABA)/glycine-activated channels prominent in the vertebrate central nervous system, and the extrajunctional glutamate/GABA activated Cl^- channels seen in both insect and crayfish muscle (16, 21). Apart from their selectivity for anions, all of these channels are of relatively small conductance (≈25-75 pS), and at least the vertebrate GABA channels (3) and the colonic epithelium Cl^- channels (34) follow halide selectivity sequence I of Wright and Diamond (39): I > Br > Cl > F.

During our studies of Cl^- channel block by crude venom and purified chlorotoxin, we have consistently seen effects only with application to what appears to be the cytoplasmic-facing surface of neuronal growth cone or colonic enterocyte Cl^- channels (11). This intracellular site of action is difficult to reconcile with the observed toxicity of chlorotoxin to insects. However, it is unlikely that epithelial Cl^- channels are the natural target for chlorotoxin. The paralysis resulting from chlorotoxin injection into arthropods may be the result of the inhibition of some other structurally related anion channel such as the extrajunctional channels of arthropod muscle, noted above. Chlorotoxin may be effective when applied to the extracellular surface of these channels.

Studies of the small-conductance epithelial Cl^- channels have been hindered by the lack of adequate ligands. The classical anion transport inhibitors 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid, and DNDS have been used but are effective only in the low micromolar range, at best. Additionally, the specificity of these inhibitors has been called into question (24, 26). Newer and more potent drugs, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (15, 23, 38) and 94-indanyloxyacetic acid (27, 28), have been developed, and the latter has even been used to purify a putative Cl^- channel. Unfortunately, both drugs induce a rapid channel block, and both are highly lipophilic, two features which limit their usefulness.

Chlorotoxin is the first reported high-affinity peptide ligand for Cl^- channels. The model we have chosen to describe chlorotoxin binding to anion channels is supported by our data. We have previously demonstrated that the block induced by crude *Leiurus* venom follows a first-order kinetic scheme (11). The open and closed duration histograms for chlorotoxin block of anion channels can also be fit by single exponential functions (Fig. 4), suggesting a first-order binding reaction. However, alternative kinetic schemes are not ruled out by our data. The model we present does allow us to estimate the affinity of chlorotoxin for Cl^- channels. In comparison with previously described ligands, chlorotoxin represents a significant advance in being water soluble and in producing channel inhibition as a result of what appear to be individual blocking events lasting 11-12 s, on average, at +20 mV and room temperature. With these properties, chlorotoxin holds promise for use in Cl^- channel purification or as a biophysical probe of channel structure.

We acknowledge the advice regarding RP-HPLC procedures given by Drs. Evelyn Stimson and H. Phon Too. Assistance with amino acid analysis and sequencing was provided by Margaret Ehrhardt of the Protein Microsequencing Lab of Brigham and Women's Hospital and William Lane and associates of the Harvard Microchemistry Facility. Dr. Chris Miller of Brandeis University is appreciated for supplying us with the ion-exchange HPLC column and especially for his probing questions. We also received help with the preparation of the manuscript from Ellen Jacobson.

This work was supported by National Institute of General Medical Sciences Grant GM-15904.

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Received 2 January 1992; accepted in final form 28 August 1992.

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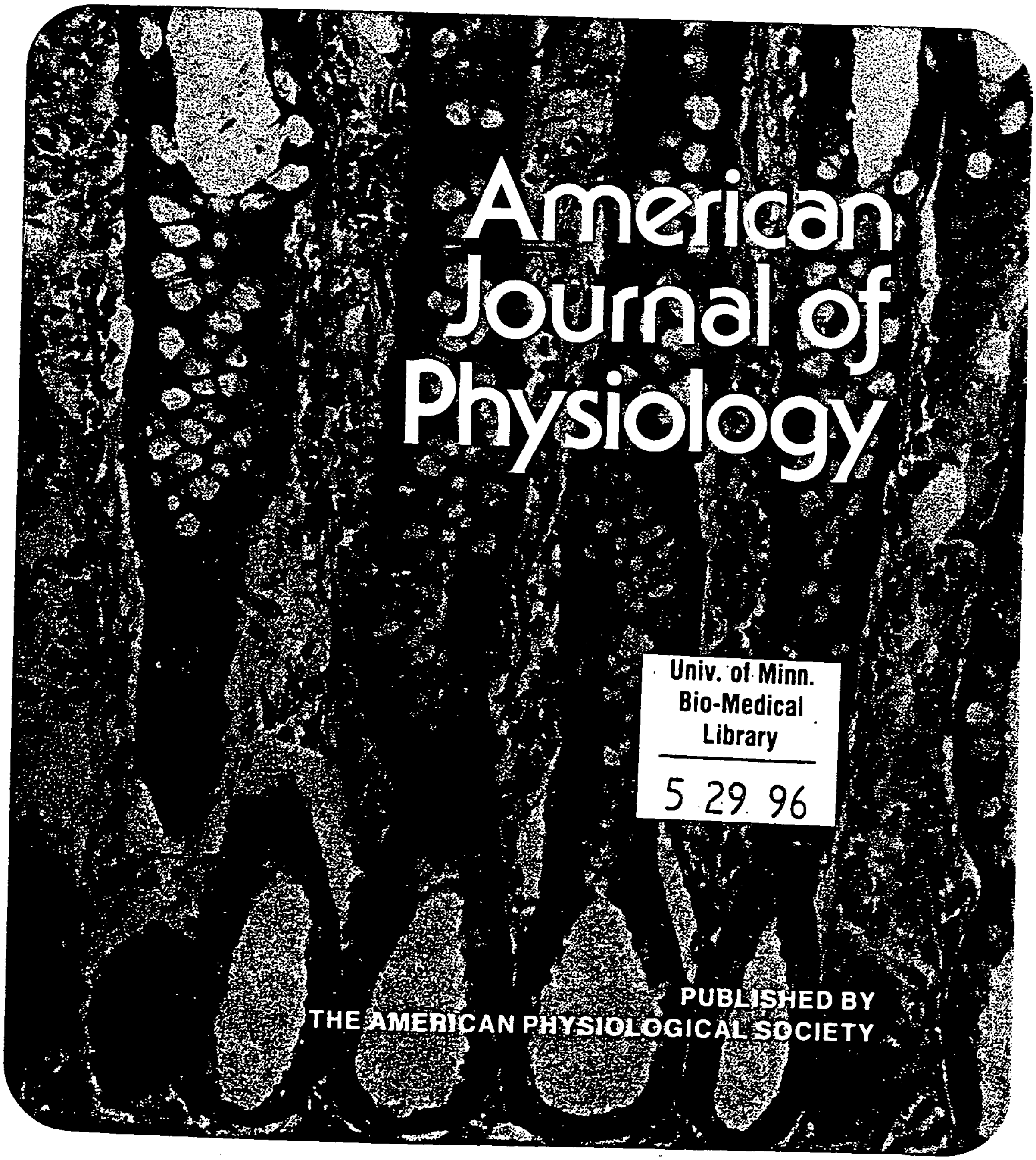
MAY 1996
Volume 270/Number 5
Part 1 of Two Parts
ISSN 0002-9513



Biophysical and pharmacological characterization of chloride currents in human astrocytoma cells

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Ullrich, Nicole, and Harald Sontheimer. Biophysical and pharmacological characterization of chloride currents in human astrocytoma cells. *Am. J. Physiol.* 270 (*Cell Physiol.* 39): C1511–C1521, 1996.—Expression of voltage-activated ion channels was studied in primary cultures from seven freshly resected human primary brain tumors and in an established human astrocytoma cell line, STTG1. Astrocytoma cells consistently expressed voltage-dependent outwardly rectifying currents. Currents activated at potentials >45 mV and showed outward transients on termination of voltage steps. Currents reversed at the Cl^- equilibrium potential, suggesting that they were largely carried by Cl^-. Altering extracellular K^+ or Na^+ concentration did not alter currents; neither did replacement of intracellular K^+ by Cs^+ or intracellular Na^+ by *N*-methyl-D-glucosamine. Anion-substitution experiments suggest the following permeability sequence, determined from shifts in tail current reversal potential: $I^- > NO_3^- > Br^- > Cl^-$ > acetate > isethionate > F^- > glutamate. Currents were sensitive to the Cl^- channel blockers chlorotoxin, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), and 4,4′-dinitrostilbene-2,2′disulfonic acid (DNDS), with chlorotoxin being most effective, yielding >80% block at 590 nM. DIDS (100 μM) and DNDS (100 μM) reduced currents by 33.5 and 38.2%, respectively. Currents were also sensitive to Zn^{2+} (100 μM, 47% block) and Cd^{2+} (25 μM, 42% block). Reducing extracellular Ca^{2+} concentration decreased outward currents by 58% and almost completely eliminated transients, suggesting that Cl^- currents are Ca^{2+} dependent. Cl^- channel block resulted in altered cell proliferation as determined by [^{3}H]thymidine incorporation, suggesting that these channels may be involved in astrocytoma growth control.

ion channel; tumor; chlorotoxin; proliferation

GLIAL CELLS COMPRISE a large proportion of the total cell population in the central nervous system. Unlike neurons, glial cells retain the ability to proliferate postnatally, and some glial cells still proliferate in the adult or aged brain (22). Uncontrolled glial proliferation can lead to aggressive primary intracranial tumors, the greatest number of which are astrocytomas and therefore of glial origin. Tumors of astrocytic origin vary widely in morphology and behavior (26) and, according to the 1993 WHO classification schema (21), can be separated into three subsets. Astrocytomas, the lowest grade tumors, are generally well differentiated and tend to grow slowly. Anaplastic astrocytomas are characterized by increased cellularity, nuclear pleomorphism, and increased mitotic activity. They are intermediate-grade tumors and show a tendency to progress to a more aggressive grade. Glioblastomas are considered the most aggressive, with poorly differentiated cells, vascular proliferation, and necrosis. Due to the common morphological heterogeneity of cells within a single tumor, such classification is not clear cut and is somewhat unsatisfactory. We collectively refer to the astrocyte-derived tumors used in our study as astrocytomas.

Recent studies suggest that ion channels may function in regulating a cell's proliferative ability. For example, mitogen-stimulated lymphocytes show an upregulation in the expression of a high-conductance K^+ channel (15). In murine fibroblasts, activation of the *ras/raf*-signaling cascade induces expression of a Ca^{2+}-activated K^+ channel that appears to be essential in the cells' proliferative response (17). The idea that ion channel expression may be necessary for cell cycle progression is also supported by observations that pharmacological blockade of ion channels can inhibit cell proliferation. This has been demonstrated in a number of cell types including melanoma (28), breast cancer cells (41), brown fat cells (30), and also several glial cell types such as Schwann cells (5), retinal glial cells (32), and astrocytes (29).

Untransformed glial cells from which glial tumors may originate have been extensively characterized electrophysiologically (37). Surprisingly, they appear to be liberally endowed with voltage- and ligand-activated ion channels for Na^+, K^+, Ca^{2+}, and possibly Cl^-. It is generally assumed that these ion channels perform homeostatic roles in the brain and may facilitate maintenance of K^+ and possibly Na^+ and Cl^- concentrations in the extracellular space. In contrast to the numerous reports on ion channel expression and activity in nonneoplastic glial cells, electrophysiological properties of astrocytoma cells and the potential role of ion channels in growth control of astrocytomas remain largely unexplored. Inwardly rectifying K^+ currents have been demonstrated in several established astrocytoma cell lines (4). We recently reported the expression of a Cl^- conductance with unique properties that appears to selectively characterize tumor-derived cells of glial origin (39).

In the present study, we have used the whole cell patch-clamp technique to characterize the biophysical and pharmacological properties of Cl^- channels in primary cultures and acutely isolated human astrocytomas. In addition, we studied an established cell line derived from a human astrocytoma. In all preparations, we observed the expression of time- and voltage-dependent outwardly rectifying currents that are sensitive to several Cl^- channel blockers and that also allow other anions to permeate. We provide evidence that this Cl^- conductance may be involved in the growth control of astrocytoma cells.

METHODS

Cell Culture

Primary cultures of human astrocytomas. Primary cultures of human astrocytomas were obtained from the University of Alabama at Birmingham Brain Tumor Research Laboratories (see Table 1 for details). Freshly resected brain tumor tissue was transported in ice-cold tissue culture medium, and necrotic-hemorrhagic portions were removed aseptically. Discrete pieces of tumor tissue were minced finely, triturated, and plated in Dulbecco's modified Eagle's medium (DMEM) mixed equally with Ham's nutrient mixture F-12 (F12) [supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 2 mM L-glutamine] with 20% fetal bovine serum (FBS; Atlanta Biologicals). Cells from minced fragments were replated onto uncoated 12-mm round coverslips for electrophysiology and for glial fibrillary acidic protein (GFAP) immmunocytochemistry. Acutely isolated tumor cells were prepared from fresh biopsy material, as described above with an additional trypsinization step to remove cellular debris, and were used for recordings 15–18 h after plating.

Cell lines. STTG1 cell line [from American Type Culture Collection (ATCC), Rockville, MD] was grown in DMEM (GIBCO) plus 10% FBS (Hyclone). Established human tumor cell lines, derived from human malignant gliomas (D54MG, U105MG, U251MG, and U373MG obtained from D. D. Bigner, Duke University) and extraglial human tumors (all from ATCC), were studied in long-term (>100) passages (see Table 1 for details). Cells were maintained in DMEM-F12 supplemented with 7% heat-inactivated FBS (Atlanta Biologicals) at 37°C in a 10% CO_2-90% air atmosphere. Cells attaining nearly confluent growth were harvested and replated onto uncoated 75-cm² flasks or uncoated 12-mm circular glass coverslips for electrophysiology and were used 36–72 h after plating, unless otherwise noted. Viable cell counts were determined by trypan blue exclusion.

GFAP immunocytochemistry. Cells were stained for expression of GFAP (GFAP monoclonal antibody from Incstar, Stillwater, MN) by use of standard immunohistochemical techniques as previously described (38).

Table 1. *Primary cultures and established astrocytoma cell lines*

Cell Line Designation	Cell Type	Passage	GFAP	Cl⁻ Current
	Primary cultures			
UAB4630	GBM	1	Unk	8/8
UAB8553	GBM	1	+	6/6
UAB12983	Low-grade astrocytoma	1	+	7/7
UAB4613	Pilocytic astrocytoma	1	+	6/6
UAB4663	Pilocytic astrocytoma	1	+	5/5
UAB4720	Anaplastic ependymoma	1	+	5/5
UAB485923	Pilocytic astrocytoma	0	Unk	10/10
	Cell Lines			
CH-235MG	GBM	>100	+	18/18
D-54MG	GBM	>100	+	11/11
SK-MG-1	GBM	>100	+	10/10
STGG1	Anaplastic astrocytoma	>100	+	470/470
U-105MG	GBM	>100	+	10/10
U-251MG	GBM	>100	+	28/28
U-373MG	GBM	>100	+	10/10

GFAP, glial fibrillary acidic protein; GBM, glioblastoma multiform; +, >70% positive; Unk, unknown.

Electrophysiology

Current (*I*) and voltage (*V*) recordings were obtained using standard whole cell patch-clamp techniques with an Axopatch-1D amplifier (Axon Instruments). Patch pipettes were made from thin-walled borosilicate glass (WPI, TW150F-40; 1.5 mm OD, 1.2 mm ID) and were filled with a solution containing (in mM) 145 KCl, 1.0 $MgCl_2$, 0.2 $CaCl_2$, 10 ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 10 HEPES [pH adjusted to 7.4 with tris(hydroxymethyl)aminomethane (Tris) unless otherwise noted]. Pipettes were not fire polished and typically had resistances between 2 and 5 MΩ. Cells were continuously superperfused with saline solution, allowing for rapid (<30 s) exchange of bath volume. The standard bath solution contained (in mM) 122.6 NaCl, 5 KCl, 1.2 $MgCl_2$, 1.0 $CaCl_2$, 2.0 Na_2HPO_4, 0.4 NaH_2PO_4, 25.0 $NaHCO_3$, 1.2 Na_2SO_4, and 10.5 glucose (bubbled with 5% CO_2-95% O_2). The composition of bath solutions used for replacement studies is summarized in Table 2. Drugs used to block ionic conductances were prepared freshly as stock solutions for each experiment and added to bath solution. Osmolality was measured with a vapor pressure osmometer (Wescor, Logan, UT) and adjusted to 308–312 mosmol/kg.

For whole cell recordings, cell capacitance compensation and series resistance compensation were used to minimize voltage errors. The amplifier reading of capacitance was used as the value for the whole cell membrane capacitance. Series resistances, monitored at regular intervals throughout each experiment, were usually 5–10 MΩ, and series resistance compensation was typically set to ~80%. Entrance potential, read from the amplifier at the time of entering the whole cell configuration, was used to determine each cell's resting potential. Voltage-clamp recordings were used to search for voltage-activated currents and stimulation profiles were altered to fully activate Cl⁻ channels (pulses from −105 to 195 mV). Where indicated, P/4 leak subtraction was obtained by use of hyperpolarizing voltage steps to obtain leak currents. Current reversal potential (*V* at which $I = 0$) was determined from *I-V* plots in which tail current amplitudes were plotted as a function of *V*. Effects of channel blockers were assessed by comparing current traces, entrance potential, and reversal potential before and after drug application. Snap photographs were taken of each recorded cell using a charge-coupled device camera and a video printer for cataloging of cell size, location, and morphology. Recordings were made at room temperature, typically 20–25°C.

Proliferation Assay

Proliferation was studied quantitatively by determining incorporation of [³H]thymidine. In brief, cells were incubated for 24 h in the continuous presence or absence of cytosine arabinoside (Ara-C, 10 μM), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS, 200 μM), 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS, 200 μM), Zn^{2+} (200 μM), or chlorotoxin (600 nM). Cells were incubated with 1 μCi/ml radiolabeled thymidine ([*methyl*-³H]thymidine) for the final 4 h (at 37°C). Culture dishes were rinsed three times with ice-cold phosphate-buffered saline and solubilized with 0.3 N NaOH for 30 min at 37°C. One aliquot (50 ml) was used for cell protein determination using the bicinchoninic acid assay (Pierce, Rockford, IL). The remaining cell suspension was mixed with Ultima Gold, and radioactivity was determined

Table 2. *Composition of external solutions (in mM)*

External Solution	Na^+	K^+	HCO_3^-	HEPES	Ca^{2+}	Mg^{2+}	EGTA	Cl^-	Br^-	F^-	I^-	NO_3^-	Isethionate	Glutamate	Acetate	Sucrose	Glucose
HCO_3^-	152	5	25		1	1.2		132									10.5
HEPES	161	5		32.5	1	1.2		132									10.5
NaBr	161	5		32.5	1	1.2		9.4	125								10.5
NaF	161	5		32.5	1	1.2		9.4		125							10.5
NaI	161	5		32.5	1	1.2		9.4			125						10.5
$NaNO_3$	161	5		32.5	1	1.2		9.4				125					10.5
Isethionate	161	5		32.5	1	1.2		9.4					125				10.5
Glutamate	161	5		32.5	1	1.2		9.4						125			10.5
Na Acetate	161	5		32.5	1	1.2		9.4							125		10.5
0 NaCl	36.5	5		32.5	1	2.2	5	9.4								250	10.5

with a scintillation counter. The results were expressed as counts per minute per microgram of protein.

Data Analysis

The theoretical equilibrium potentials were calculated according to the Nernst equation. The ion activities were adjusted from the ion concentrations used in solutions with activity coefficients obtained from Robinson and Stokes (34): 0.888, 0.886, and 0.888 for Na^+, K^+, and Cl^- concentrations, respectively. Calculated equilibrium potentials under the imposed ionic gradients in control solution were $E_K = -83.4$ mV, $E_{Na} = +62.6$ mV, and $E_{Cl} = +2.8$ mV. For all experiments, means ± SD were computed from raw values entered into a spreadsheet (Excel, Microsoft). These data were exported to a scientific graphing and data analysis program (Origin, MicroCal). Data were graphed as means ± SE. Statistics were computed from raw data. For physiological effects of channel blockers, we used a paired one-tailed *t*-test. For proliferative effects of channel blockers, results were analyzed using analysis of variance test for multiple comparisons.

Drugs Used

DIDS, DNDS, Ara-C, and all other drugs were all purchased from Sigma. Chlorotoxin was purchased from Lanoxin (Accurate Chemical and Scientific, Westbury, NY).

RESULTS

Whole cell voltage-clamp recordings were obtained from primary cultures of seven freshly resected primary human brain tumors. In addition, a human anaplastic astrocytoma cell line, STTG1, was studied. Most of the STTG1 and primary-cultured cells were GFAP positive. Cells chosen for recordings were typically alone or isolated from other cell clusters and displayed bipolar fibroblast-like morphology. Under normal recording conditions, we observed time- and voltage-dependent outward currents in all (n = 490) recorded STTG1 astrocytoma cells and all recorded primary cultured astrocytoma cells (n = 60). We also obtained recordings from acutely isolated tumor cells within 15–18 h of plating (UAB485923, n = 10). Currents were qualitatively similar in all preparations. The resting potential, determined as the entrance potential with KCl-containing pipette solution, was −14.1 mV (n = 490, SD = 14.6, SE = 0.66) and −20.15 mV (n = 60, SD = 17.54, SE = 2.28) in cell lines and primary cultures, respectively.

Cl^- Currents in Human Astrocytoma Cells

Representative examples of whole cell recordings from an STTG1 human astrocytoma cell and an astrocytoma cell from primary culture (UAB4630) in response to depolarizing voltage steps are displayed in Fig. 1. The cells were stepped from a holding potential of 0 mV to a series of test potentials between −105 and 195 mV in 25-mV increments. Potential of >45 mV resulted in fast-activating noninactivating outward currents. Cells showed large outward transients on termination of voltage steps (Fig. 1, *A* and *C*). The *I-V* relation plotting peak current amplitude as a function of voltage (Fig. 1, *B* and *D*) showed pronounced voltage dependence and outward rectification for both the transients (Fig. 1, *B* and *D*, *) and steady-state currents (Fig. 1, *B* and *D*, ×). Mean conductance of 36 primary cultured cells was 5.67 nS (SD = 4.62, SE = 0.77) and of 50 STTG1 cells was 5.29 nS (SD = 3.63, SE = 0.51; determined at 145 mV). To account for differences in cell size, we normalized values to membrane capacitance, yielding specific conductances of 195 and 208 pS/pF, respectively. To determine the ion species that was carrying the outward current, we analyzed the reversal potential of tail currents. Therefore cells were held at 0 mV, pulsed to 180 mV, and then stepped in −20-mV increments from +120 to −120 mV (Fig. 2*A*). Plotting tail currents as a function of voltage showed a reversal potential of 8 mV (Fig. 2*B*) in this example. Analysis of 12 primary cultured cells yielded a mean reversal potential of 0.1 (SD = 11.3) and −4.6 mV (n = 48, SD = 14.1) in STTG1 cells. Under the imposed ionic gradients ($E_{Cl} = +2.8$, $E_K = -83.4$, $E_{Na} = +62.6$ mV), this is compatible with a reversal potential expected for either a Cl^--selective or a nonselective cation current. Cells from all studied primary cultures and all STTG1 cells displayed such outwardly rectifying currents, and subsequent analysis did not distinguish between these two preparations.

Channel Selectivity for Cl^-

To determine the ion selectivity of the outward current, we substituted all but 9.4 mM of the Cl^- in the bath solution with the sodium salts of a number of other monovalent anions (see Table 2 for composition of solutions) while keeping the pipette Cl^- concentration constant (147.4 mM). To facilitate ion-replacement studies, we used HEPES-buffered solutions; changing

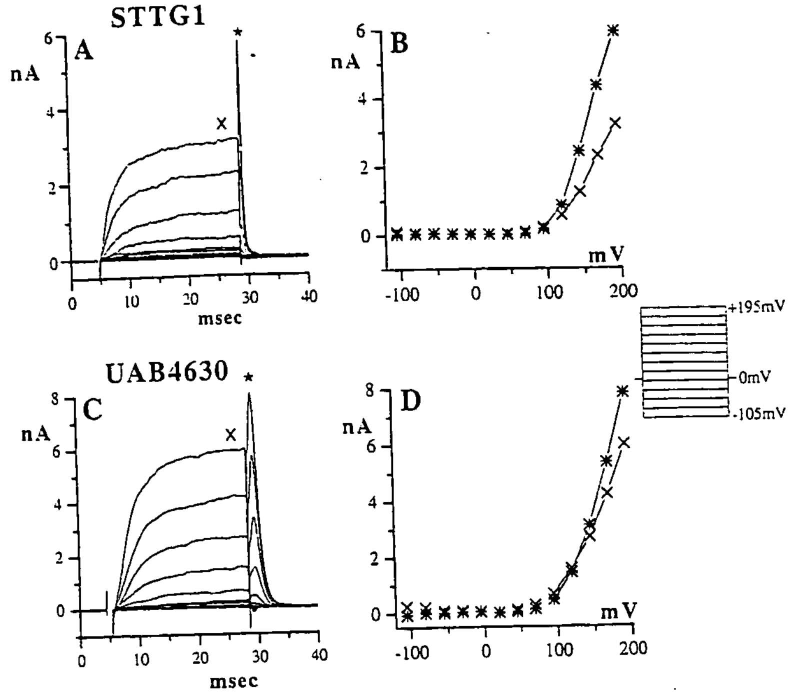

Fig. 1. Whole cell voltage-clamp recordings obtained from a representative human astrocytoma cell from cell line STTG1 and from a primary cultured astrocytoma cell (UAB4630). Cells were stepped to test potentials between −105 and 195 mV in 25-mV increments from a holding potential of 0 mV (*inset*). Cells showed large transients on termination of voltage steps (*, *A* and *C*). Potential of >45 mV resulted in fast-activating noninactivating outwardly rectifying currents (*B* and *D*).

to HEPES-buffered solution compared with HCO_3^--buffered solution by itself did not alter currents, suggesting that HCO_3^- does not permeate the channel under these conditions. Recordings obtained in HEPES- and HCO_3^--buffered external solutions were virtually indistinguishable, with no change in current amplitude or tail current reversal potential (data not shown).

Figure 3 shows examples of whole cell leak-subtracted current responses of human astrocytoma cells to test pulses stepped from a holding potential of 0 to 145 mV before and after substitution of bath Cl^- with the halide anions Br^- (*A*), I^- (*B*), NO_3^- (*C*), and F^- (*D*). Br^-, I^-, and NO_3^- increased outward currents, whereas F^- substitution led to decreased currents. For each experiment, complete *I-V* curves were plotted in Fig. 3*E*. To compare *I-V* relations, currents were normalized to control currents with Cl^- as the external anion as the membrane was stepped from 0 mV to a series of potentials between −105 and +195 mV. Largest currents in Cl^--containing control solution were arbitrarily defined as 1. Currents in I^- and NO_3^- exceeded Cl^- currents by greater than twofold. Similarly, Fig. 4 shows the whole cell leak-subtracted current responses with the same experimental protocol as in Fig. 3 before and after substitution with acetate (*A*), glutamate (B), isethionate (*C*), and sucrose (*D*). Acetate and isethion-

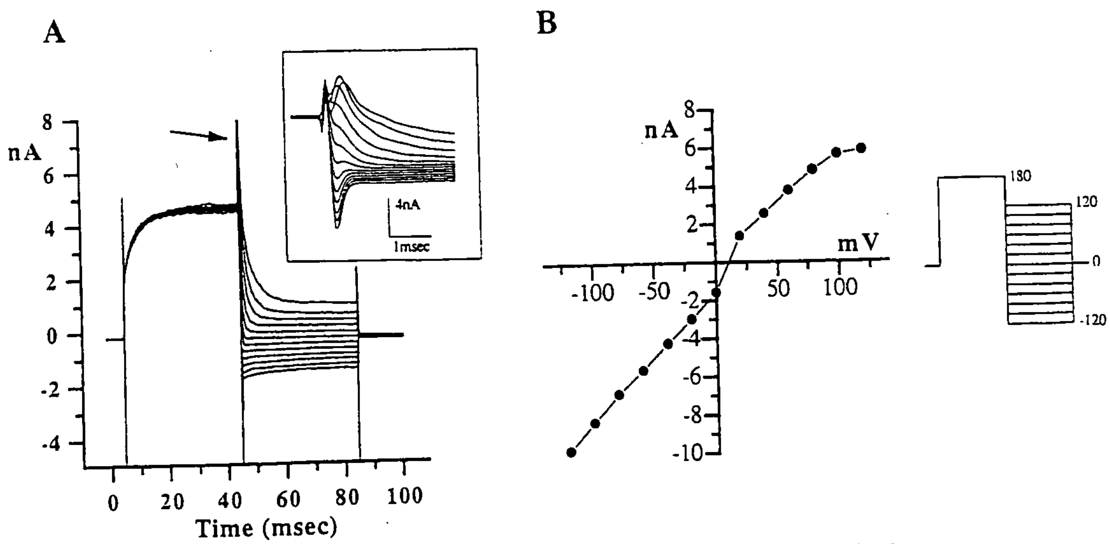

Fig. 2. To determine ion species that was carrying outward current, we analyzed reversal potential of tail currents. Cells were held at 0 mV, pulsed to 180 mV, and then pulsed in −20-mV steps from +120 to −120 mV (*A*, *inset*). Plotting tail current amplitudes as a function of voltage showed a reversal potential of 8 mV (*B*).

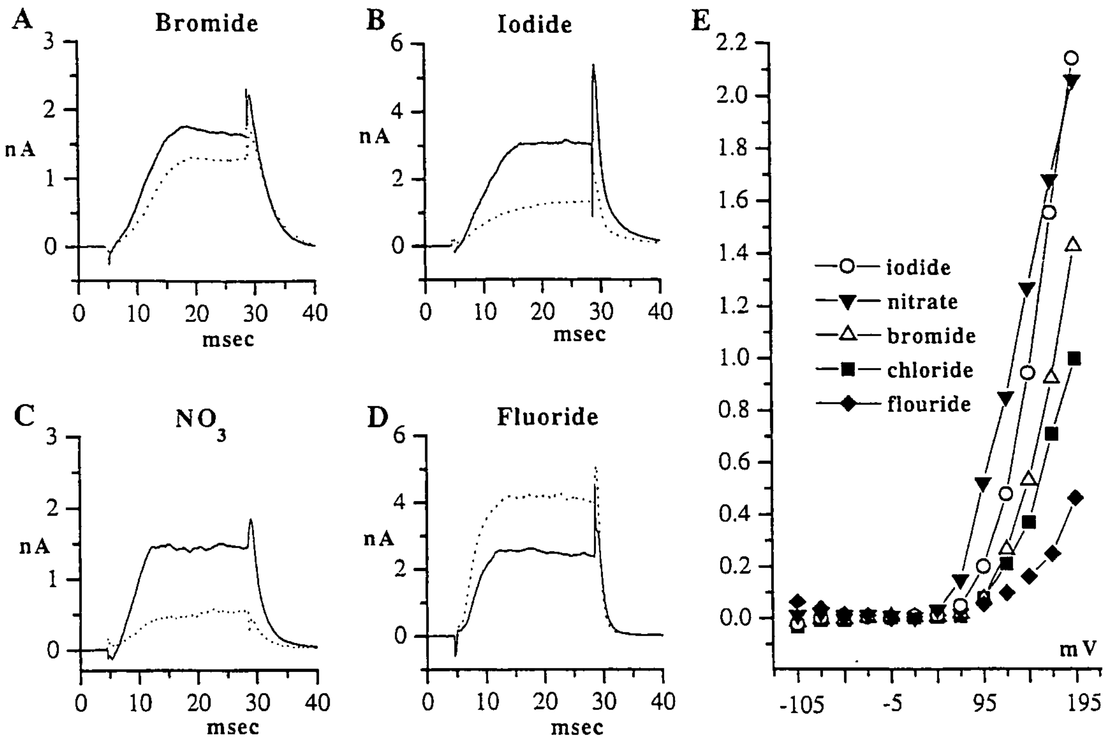

Fig. 3. Whole cell leak subtracted current responses of STTG1 cells in response to a single 145-mV voltage step before and after substitution of extracellular Cl^- with 125 mM Br^- (*A*), I^- (*B*), NO_3^- (*C*), or F^- (*D*). Dashed lines represent control current with standard external solution, and straight lines represent current with replacement solution. *E*: peak current-voltage (*I-V*) relations obtained as in Fig. 1, with current normalized to that obtained with standard NaCl-rich external solution.

ate led to decreased outward currents, whereas glutamate and sucrose virtually eliminated outward currents. The *I-V* relations for the nonhalide substitutions normalized to normal NaCl-rich bath solution are shown in Fig. 4*E*.

The selectivity for the different anions was calculated from the shift of the reversal potential (ΔR_{rev}) under the imposed ionic gradients according to the Goldman-Hodgkin-Katz equation

$$\Delta E_{rev} = E_{rev,anion} - E_{rev,Cl}$$
$$= (RT/zF)\ln(P_{anion}[anion]_o/P_{Cl}[Cl]_o$$

where R, T, and F have their usual meanings and z is

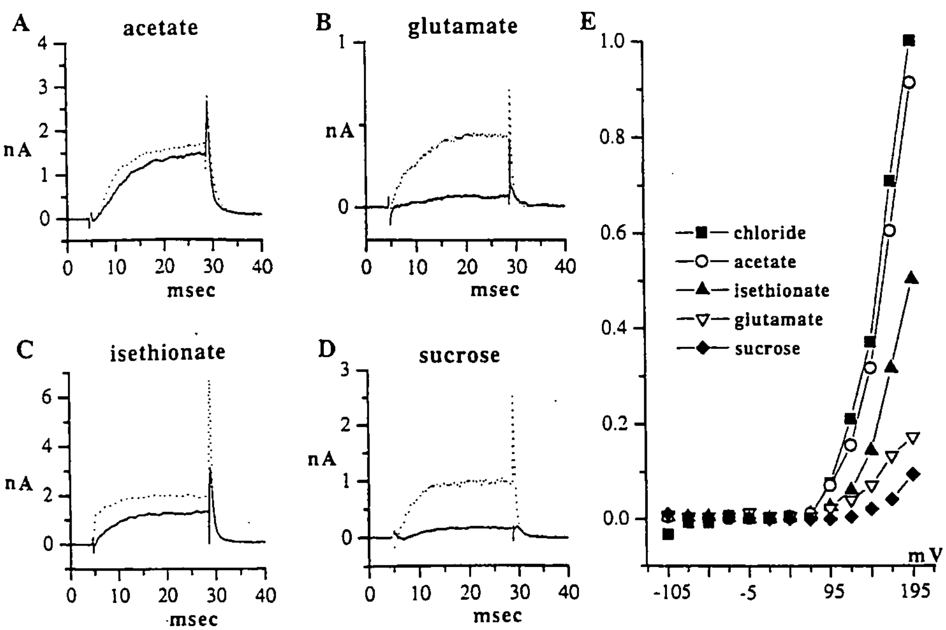

Fig. 4. As in Fig. 3, whole cell leak subtracted current responses of STTG1 cells before and after substitution of extracellular Cl^- with 125 mM acetate (*A*), glutamate (*B*), isethionate (*C*), or sucrose (*D*). As above, dashed lines represent control current with standard bath solution, and straight lines represent current after replacement. *E*: peak *I-V* relations, normalized to current in presence of 125 mM Cl^- as above.

Table 3. *Anion selectivity*

Na^+ Anion	MW	ΔE_{rev}, mV	P_{anion}/P_{Cl}	*n*
Chloride	58.45			48
Acetate	82.04	4.0 ± 0	0.90 ± 0.02	3
Bromide	102.9	-15.5 ± 6.3	1.95 ± 0.47	3
Fluoride	42.0	24.6 ± 3.0	0.41 ± 0.05	3
Glutamate	169.1	29.8 ± 2.3	0.33 ± 0.18	2
Iodide	149.9	-20.8 ± 7.5	2.44 ± 0.65	5
Isethionate	148.1	18.5 ± 0.7	0.51 ± 0.01	3
Nitrate	85.01	-15.8 ± 8.8	2.02 ± 0.58	4
(Sucrose)	342.3	32.2 ± 17.8		3

Values are means ± SE. Reversal potential (E_{rev}) was determined in each test solution by plotting peak tail current amplitude against applied voltage step after cells were stepped to 180 mV and then brought from +120 to −120 mV in −20-mV increments. Results are expressed as mean change in E_{rev} from control solution containing NaCl. Permeability ratio P_{anion}/P_{Cl} was determined using the Goldman-Hodgkin-Katz equation. MW, molecular weight.

the valence. In this calculation, we assumed that the currents measured under the conditions of the experiment were carried solely through Cl^- channels. In total the permeability of seven different anions was tested. Table 3 summarizes the changes in the values of E_{rev} for equimolar replacement of Cl^- by test anions and the calculated permeability ratios (P_{anion}/P_{Cl}). These data suggest the following relative permeability sequence: $I^- > NO_3^- > Br^- > Cl^- >$ acetate > isethionate > $F^- >$ glutamate.

Effect of Cl^- Channel Blockers

We further characterized the outward current pharmacologically by examining the effect of several established Cl^- channel blockers, including chlorotoxin, DIDS, and DNDS.

Figure 5 shows representative whole cell leak-subtracted traces and *I-V* relations before and after bath addition of chlorotoxin, DIDS, and DNDS. Bath application of 590 nM chlorotoxin reduced both the

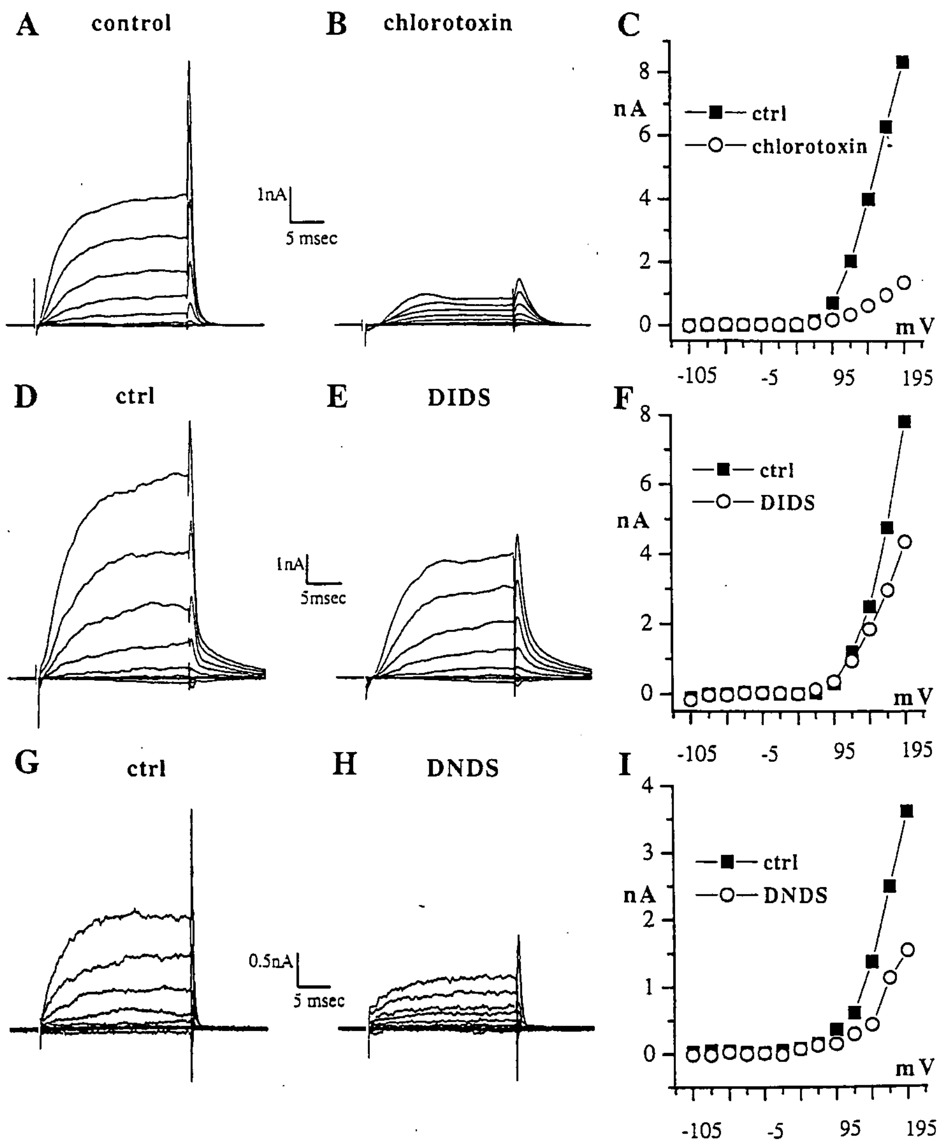

Fig. 5. Effect of bath application of chlorotoxin, DIDS, and 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) on outward currents in STTG1 astrocytoma cells in response to test voltage pulses from −105 to +195 mV in 25-mV increments. Whole cell currents are shown before (*A*) and after (*B*) bath application of 590 nM chlorotoxin. Chlorotoxin decreased outward currents by 81%. *C*: *I-V* relation of peak current amplitude as a function of applied voltage. Currents are also shown before and after application of 100 μM DIDS (*D* and *E*) and 100 μM DNDS (*G* and *H*). *I-V* relations from those examples are shown in *F* and *I*. Size of outward current is reduced by DIDS at all potentials (33.5 ± 12.9%, *n* = 5). Similar to effect of DIDS, DNDS caused a decrease in current amplitude at all potentials by 38.2 ± 13.3% (*n* = 4). Ctrl, control.

steady-state and transient current amplitudes evoked by voltage steps from −105 to +195 mV by 81.9 ± 0.88% (n = 4) of the control value (Fig. 5, A–C). This effect was partially reversible. Chlorotoxin was also effective at higher concentrations in blocking Cl^- currents when applied to the cytoplasmic face (60.44% at 2.5 µM intracellular chlorotoxin, n = 7, SD = 17.8, data not shown). Chlorotoxin is a 36-amino acid scorpion venom toxin that was originally described as a blocker of small-conductance Cl^- channels in epithelial cells (8). To ensure that the effects of chlorotoxin did not result from any contaminants in the venom toxin, we synthesized the peptide and observed comparable inhibition of currents with the synthetic toxin (data not shown). As above, currents are shown before and after application of 100 µM DIDS (Fig. 5, D and E) and 100 µM DNDS (Fig. 5, G and H). I-V relations from those examples are shown in Fig. 5, F and I. The size of the outward current was reduced by DIDS at all potentials (33.5 ± 12.9%, n = 5). Similar to DIDS, DNDS caused a decrease in current amplitude at all potentials by 38.2 ± 13.3% (n = 4). DIDS and DNDS were more effective in blocking currents when applied to the cytosolic face, although at higher concentrations (200 µM, 50 ± 10.9%, n = 3, and 62%, n = 1, respectively, data not shown). The action of both drugs was partly reversible with short exposure times, although the recovery was never complete.

We also examined the effects of the heavy metals Zn^{2+} and Cd^{2+} on outward currents. These drugs have been shown to block Cl^- currents in T lymphocytes (35) and Schwann cells (33). Bath application of 100 µM Zn^{2+} led to a 47 ± 25.9% (n = 3) decrease in peak currents (Fig. 6, A–C), and 25 µM Cd^{2+} led to a 42 ± 18.5% (n = 5) decrease (Fig. 6, D and E). Because Cd^{2+} is also a blocker of voltage-dependent Ca^{2+} channels, it is possible that reduced currents may have resulted indirectly from reducing Ca^{2+} influx. To help elucidate whether this may have been the case, we applied bath solution in which all Ca^{2+} had been removed and with the addition of 5 mM EGTA. In a zero Ca^{2+} environment, currents were decreased to 42.6 ± 16.8% (n = 5)

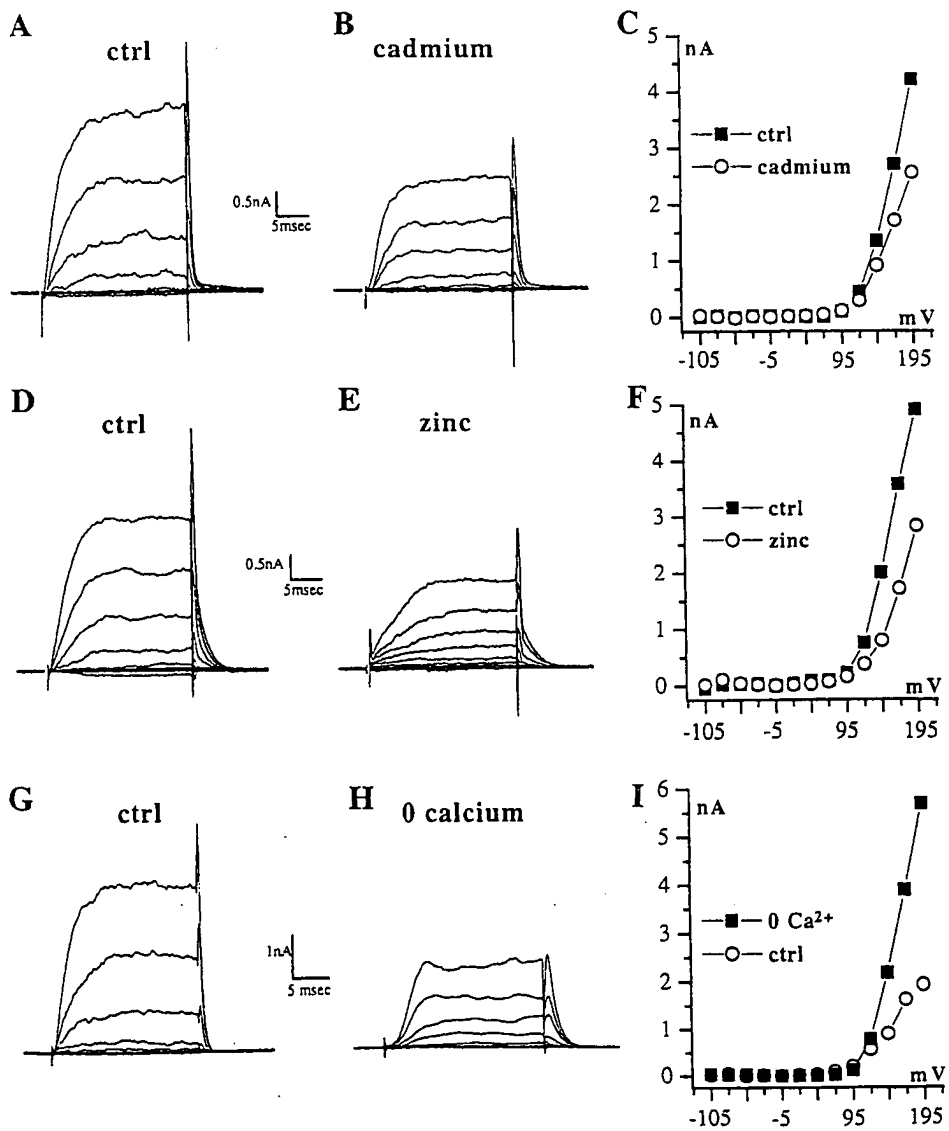

Fig. 6. Effect of Zn^{2+}, Cd^{2+}, and Ca^{2+} on outward currents. Bath application of 100 µM Zn^{2+} led to a 47 ± 25.9% (n = 3) decrease in peak currents (A–C), and 25 µM Cd^{2+} led to a 42 ± 18.5% (n = 5) decrease (D and E). In bath solution with 0 Ca^{2+} and 5 mM EGTA, currents were decreased to 40% of that in control solution containing 1 mM Ca^{2+} (F–H).

of that in control solution containing 1 mM Ca^{2+} (Fig. 6, *F–H*), suggesting that Cl^- currents are indeed at least partially dependent on extracellular Ca^{2+} concentration. A summary of the pharmacological effects on current amplitude is shown in Fig. 7, with the values expressed as percentage of control current in standard external solution.

On the basis of the ion-replacement studies and pharmacology, we conclude that the outwardly rectifying currents were mediated by anions. Under physiological conditions, the current would be carried by Cl^-; thus we refer to it as a Cl^- current.

Cl^- Channels and Astrocytoma Proliferation

Given that these Cl^- currents were consistently present in all astrocytoma cells tested from both primary cultures of surgical specimens and from established human astrocytoma cell lines, we were interested to know whether Cl^- currents influence astrocytoma proliferation. The effects of Cl^- channel blockade on cell proliferation have been reported in Schwann cells (40) and B lymphocytes (7). We cultured cells in the continuous presence of the antimitotic agent Ara-C (10 mM), DIDS (200 µM), DNDS (200 µM), Zn^{2+} (200 µM), or chlorotoxin (600 nM) and compared the rate of proliferation to untreated (control) sister cultures (Fig. 8). Cells were treated at 2 days in culture and proliferation was assayed 24 h later, at 3 days in culture, a period of high proliferation of untreated control cultures. As expected, incubation in Ara-C led to a 70% decrease in proliferation (SD = 1.3309, n = 17). The putative Cl^- channel blockers DIDS, DNDS, and Zn^{2+} decreased proliferation by 16.4% (SD = 20.0, n = 16), 38.2% (SD = 13.1, n = 8), and 72.6% (SD = 12.4, n = 7), respectively. By contrast, incubation in either the native or synthetic venom toxin chlorotoxin led to an increase in proliferation compared with control (37.8%, SD = 5.7, n = 8 and 28.4%, SD = 16.34, n = 9, respectively).

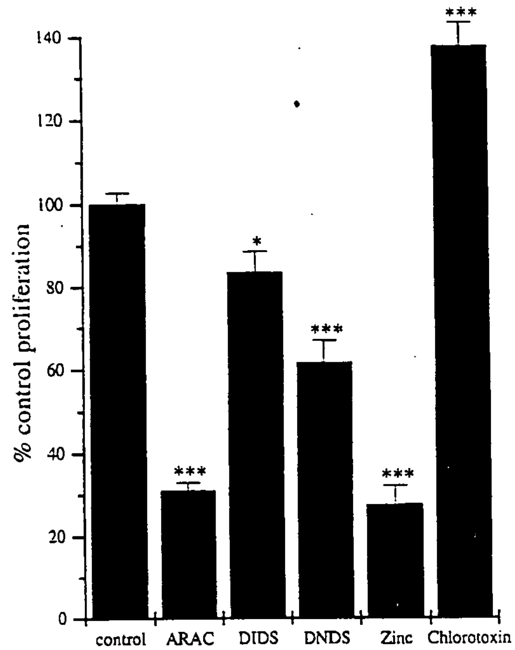

Fig. 8. Effects of antimitotic agent, cytosine arabinoside (Ara-C, 10 mM), DIDS (200 µM), DNDS (200 µM), Zn^{2+} (200 µM), and chlorotoxin (600 nM) on astrocytoma proliferation, assessed as [^{3}H]thymidine incorporation after 24 h of incubation with agent of interest. Mean effects (expressed as cpm/µg protein; error bars, SD) were plotted for each agent tested in at least 6 experiments each (see text for details). As expected, incubation in Ara-C led to a 70% decrease in proliferation (SD = 1.3309, n = 17). Cl^- channel blockers DIDS, DNDS, and Zn^{2+} decreased proliferation by 16.4% (SD = 20.0, n = 16), 38.2% (SD = 13.1, n = 8), and 72.6% (SD = 12.4, n = 7), respectively. By contrast, incubation in chlorotoxin led to a 37.8% increase in proliferation compared with control (SD = 5.7, n = 8). Error bars reflect SE.

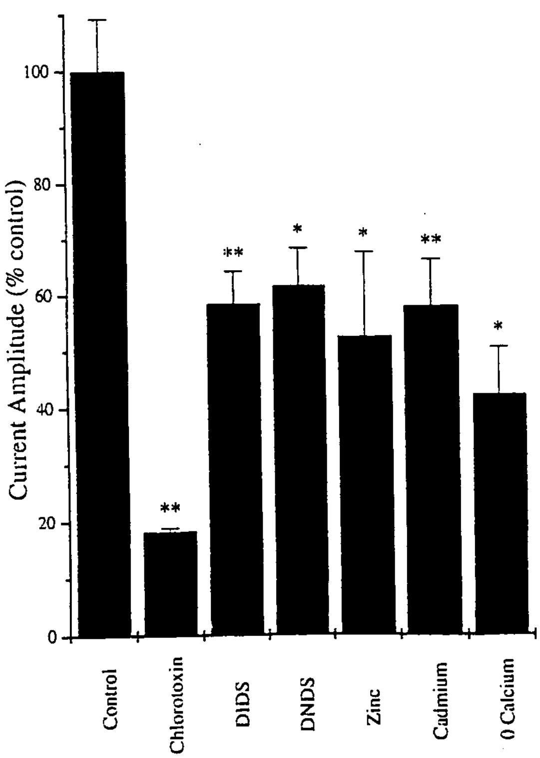

Fig. 7. Comparison of effects of channel blockers on outward currents. Effects are expressed as percentage of normalized to current amplitude obtained in standard NaCl-rich external solution for pooling of experimental data. Error bars reflect SE.

DISCUSSION

We have identified a voltage-dependent outwardly rectifying Cl^- current in human astrocytoma cells. This current was present in all cells studied in both primary cultures of human astrocytomas and in an established human astrocytoma cell line. Cells showed large outward transients on termination of voltage steps and reversed close to the calculated equilibrium potential for Cl^-. On replacement with various anions, the current reversal potential shifted in accordance with an anion-selective channel toward the new E_{Cl}. Currents were sensitive to application of Cl^- channel blockers chlorotoxin, DIDS, DNDS, Cd^{2+}, and Zn^{2+}. Under physiological conditions, the current would be carried by Cl^-, so that currents were considered Cl^- currents. The presence of the current was surprising in light of the

fact that nonneoplastic glial cells are typically characterized by high levels of expression of voltage-gated K^+ channels; we did not observe any appreciable contribution from K^+ currents to whole cell outward currents in all cells tested.

Outwardly rectifying Cl^- currents have been described in many epithelial tissues including respiratory cells (24), submandibular gland (19), lacrimal gland (9), pancreatic duct cells (18), epididymis (31), and sweat gland (23) and in nonepithelial cells such as lymphocytes (11), squid axon (18), and rat skeletal muscle (3). The physiological function of these outwardly rectifying channel in cell types other than secretory epithelia remains unclear. In the latter, they are believed to participate in transepithelial solute transport and volume regulation (10).

The current observed in astrocytoma cells, although similar to epithelial cells in its sensitivity to Cl^- channel blockers, shows several differences. *1*) In some preparations, such as fetal pancreas (13), fetal epididymis (31), and pancreatic ductal cells (2), Cl^- currents show little or no voltage dependence. *2*) Another class of Cl^- channels shows a peculiar voltage dependence, with activation near 0 mV and inactivation with potentials of >20 mV in either direction (3, 27, 36). Astrocytoma Cl^- channels are strongly voltage dependent at all potentials of >50 mV. In this regard, they are most similar to Cl^- channels found in human macrophages (16), *Necturus* enterocytes (12), squid axon (18), and sheep parotid gland (19). *3*) In some cell types, such as colon muscle (1), submandibular gland (19), rat muscle (3), and A6 epithelia cells (27), Cl^- channels do not show spontaneous activity in whole cell recordings, and channel activation occurs only in excised patches. In contrast, astrocytoma Cl^- currents could be easily recorded in every recording in the whole cell configuration.

The permeability sequence of the Cl^- channel in astrocytoma cells does not correlate with the hydrated ion radii ($NO_3^- > Cl^- > I^- > Br^-$) or the mobility of ions in aqueous solution ($Br^- > I^- > Cl^- > NO_3^-$). The sequence most closely resembles the lyotrophic series ($I^- > NO_3^- > Br^- > Cl^- > F^-$), which reflects the ability to denature macromolecules or to bind or absorb to proteins or lipid-water interfaces (6). The anion selectivity sequence here differs in only minor detail from those reported for outwardly rectifying channels in other tissues: submandibular duct gland ($SCN^- > NO_3^- > I^- > Cl^- > Br^- >$ acetate) (19), canine airway epithelia ($SCN^- > NO_3^- > I^- Br^- > NO_3^- > Cl^-$) (25), rat lacrimal gland ($I^- > NO_3^- > Br^- > Cl^- > F^- >$ isethionate > glutamate) (9), and *Necturus* enterocytes ($SCN^- > I^- > Br^- > Cl^- > F^- >$ gluconate) (12). Typically, replacement of Cl^- by large organic anions results in the virtual abolishment of Cl^- currents. Similarly, in our recordings, currents were almost eliminated after glutamate or sucrose replacement.

Brismar and Collins (4) tested various human astrocytoma cell lines and found a high density of inwardly rectifying K^+ channels (K_{ir}) active at or near resting potential. The current component active at potentials more negative than 0 mV was blocked by Cs^+ and was dependent on extracellular K^+, such that replacement with high-K^+ solutions led to an increase in the inward currents. On closer examination, the current component active at potentials more positive than 0 mV was insensitive to ion replacements of Na^+ or K^+ and was also insensitive to Cs^+ blockade. This is the range of voltage steps that produces an *I-V* relation most similar to the one we observed. These authors did not further investigate the current contributions >0 mV. We did not see any appreciable contribution of K_{ir} currents in our studies. It is possible that the cells' proliferative state or differences in culture conditions may alter the presence of K_{ir} and may account for the differences between their and our studies. However, unlike Brismar and Collins (4), we also studied cells prepared from primary cultures of surgical specimens from astrocytomas and did not see any appreciable K_{ir} currents.

Recently, a Cl^- current has been described in an astrocytoma cell line (U373MG) that is only activated by hyposmotic conditions but not present under normosmic conditions (1). These authors report that outward currents are sensitive to one of the Cl^- channel blockers used in our experiments, namely DIDS, in addition to some additional putative channel blockers. Although the *I-V* relations appear similar to those we have described in the same voltage range, currents do not show the large outward transients on termination of the voltage steps characteristic in our experiments. Most importantly, the currents in these cells were not active under normosmic conditions, and the cells must have been exposed to hyposmotic bath solutions before the Cl^- currents could be evoked. Again, these authors did not investigate cells from surgical specimens in their studies. In addition, the presence of an anion current in cultured rat cortical astrocytes has been recorded which is active only in 1–2 of 100 excised patches in normosmic conditions and with increased frequency in hyposmic conditions (20). Whole cell Cl^- currents were previously recorded in cultured rat astrocytes; however, these currents differ markedly in their voltage dependence and relative permeability to anions from that described here (14). We have previously reported the presence of outwardly rectifying voltage-dependent Cl^- currents in biopsies prepared from surgical specimens from six different human astrocytomas and seven different established human astrocytoma cell lines (39) under normosmic conditions. In our hands, we were able to record similar currents in the cell line U373MG, used in the aforementioned study, in addition to other established astrocytoma cell lines (CH-235MG, D-54MG, SK-MG-1, U-105MG, U251MG; see Table 1 for details, data not shown). Moreover, the currents were observed in all cells under normal conditions, with osmolality of each solution measured and matched to the osmolality of the growth medium. The discrepancy between these results needs further investigation.

The precise role of this Cl^- conductance in astrocytoma cells is unclear. Ion channels have been shown to be part of the proliferative response in a number of cell

types, and we have previously shown, in cultures of normal glial cells, that the activity of K^+ channels is required for cell proliferation, since K^+ channel blockade leads to decreased proliferation (29). K^+ channels have been implicated in the proliferative response in a number of other cell types, including human melanoma cell lines (28), cultured brown fat cells (30), and Schwann cells, the principal glial cells in the peripheral nervous system (5). This suggests that the link between channel activity and proliferation is more widespread. Modulation of channels may result from both long-term changes in gene expression and short-term modulation of preexisting channel proteins.

A link between Cl^- channels and the proliferative response has only recently been suggested. In cultured B cells, the stilbene disulfonates and putative Cl^- channel blockers 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) and DIDS were found to be effective mitogens and directly stimulated proliferation (7). Moreover, the mitogenic responses to DIDS were routinely larger than those obtained with the B cell mitogen lipopolysaccharide. These experiments imply that there is a signal transduction pathway leading to cell proliferation that directly involves anion movement across the cell membrane. In Schwann cells, SITS and DIDS application leads to a two- to fivefold enhancement of proliferation in both unstimulated and mitogen-stimulated proliferation (40). We observed a decrease in proliferation by DIDS, DNDS, and Zn^{2+} and a 37% enhancement of astrocytoma proliferation after application of chlorotoxin. One possible explanation is that the stilbene derivatives are affecting ion transport mechanisms, whereas chlorotoxin is a more specific ion channel inhibitor. Despite these disparate effects, these results suggest that Cl^- channels may participate in the proliferative response in these cells and we are currently exploring this possibility further.

We thank G. Yancey Gillespie for supplying the primary brain tumor cultures.

This work was supported by National Institutes of Health Grants RO1-NS-31234 and P50-HD-32901.

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Received 21 September 1995; accepted in final form 13 November 1995.

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US006429187B1

(12) **United States Patent**
Sontheimer et al.

(10) **Patent No.: US 6,429,187 B1**
(45) **Date of Patent: *Aug. 6, 2002**

(54) **METHOD OF DIAGNOSING AND TREATING GLIOMAS**

(75) Inventors: **Harald W. Sontheimer**, Birmingham, AL (US); **Nicole Ullrich**, Fairfield, CT (US)

(73) Assignee: **UAB Research Foundation**, Birmingham, AL (US)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/980,395**

(22) Filed: **Nov. 28, 1997**

Related U.S. Application Data

(62) Division of application No. 08/774,154, filed on Dec. 26, 1996, now Pat. No. 5,905,027.
(60) Provisional application No. 60/009,283, filed on Dec. 27, 1995.

(51) **Int. Cl.**[7] **A61K 38/00**

(52) **U.S. Cl.** **514/2**; 530/350; 424/130.1; 424/1.49

(58) **Field of Search** 435/7.23; 424/1.11, 424/1.41, 1.49, 130.1; 514/2; 530/350

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Primary Examiner—Sheela Huff
(74) *Attorney, Agent, or Firm*—Morgan, Lewis & Bockius LLP

(57) **ABSTRACT**

The present invention provides a recombinant toxin and monoclonal antibody which specifically binds to glial-derived or meningioma-derived tumor cells. Also provided are various methods of screening for malignant gliomas and meningiomas. Further provided are methods of treating malignant gliomas, including glioblastoma multiforme and astrocytomas.

9 Claims, 20 Drawing Sheets

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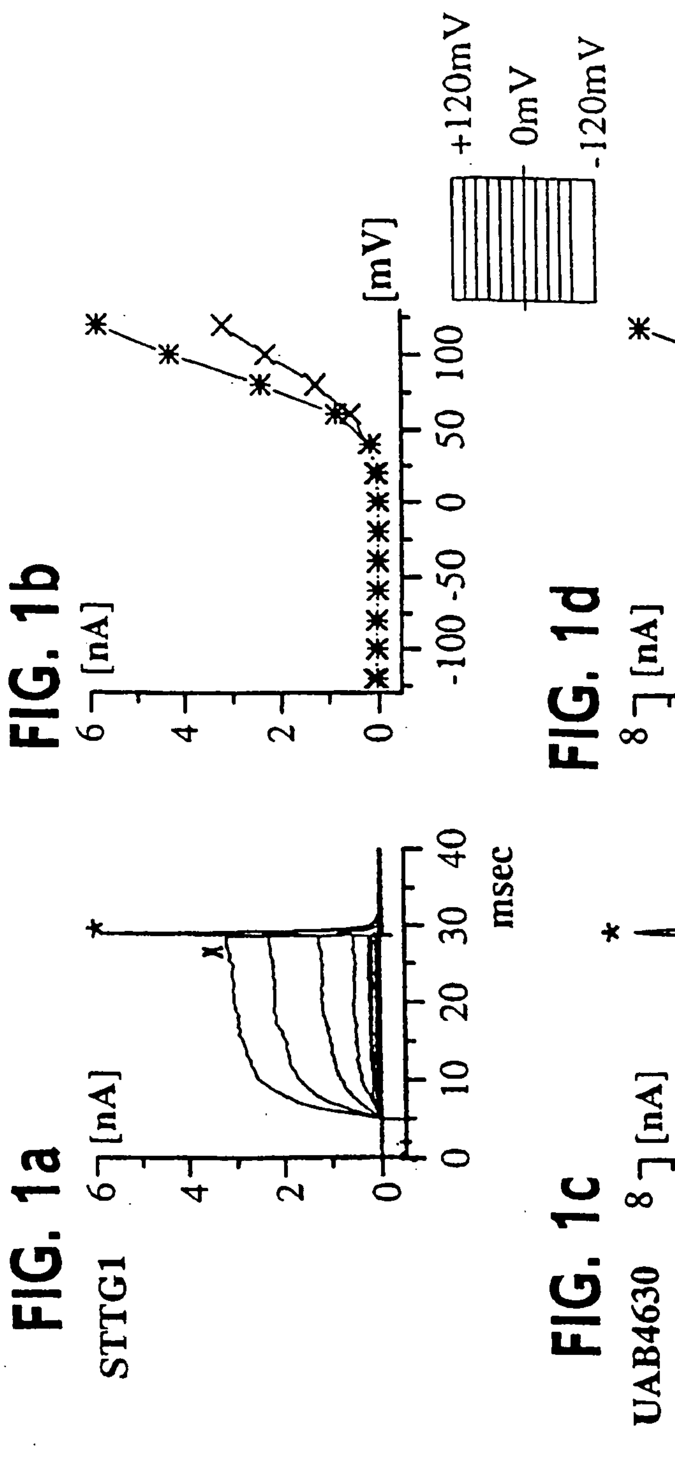


FIG. 1a

FIG. 1b

FIG. 1c

FIG. 1d

FIG. 2b

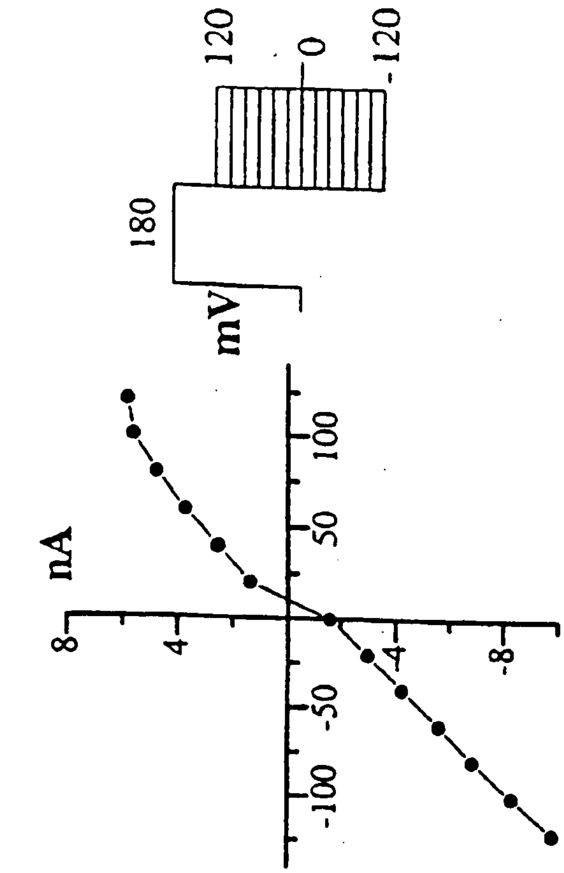

nA
8
4
-4
-8
-100
-50
50
100
mV
180
120
0
-120

FIG. 2a

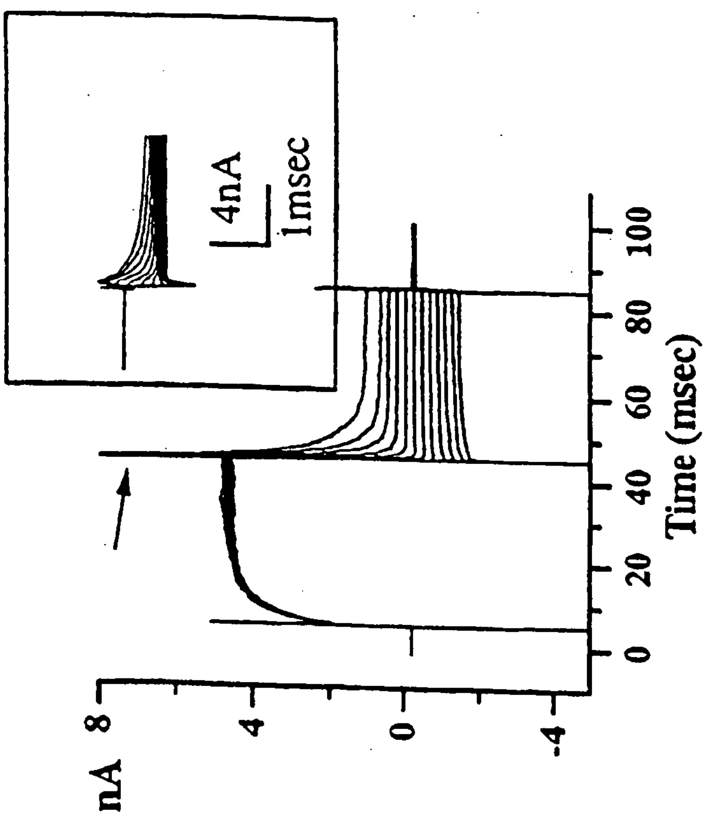

nA
8
4
0
-4
0
20
40
60
80
100
Time (msec)
4nA
1msec

FIG. 3e

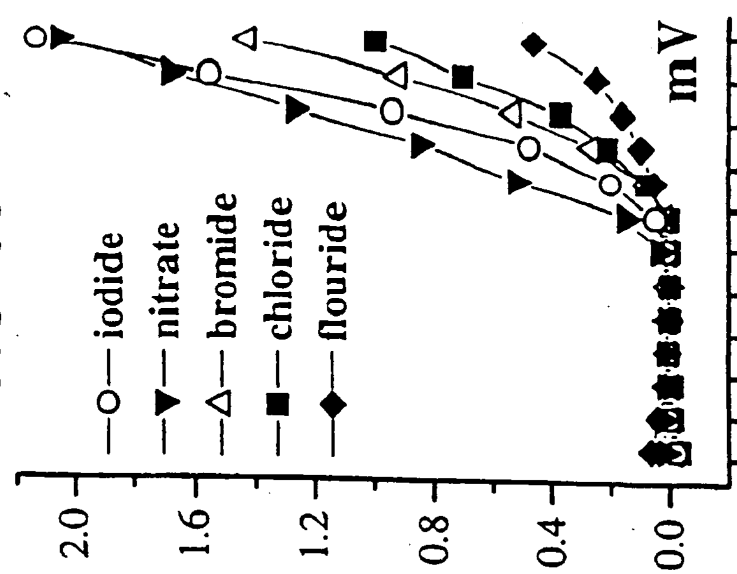


FIG. 3b
Iodide

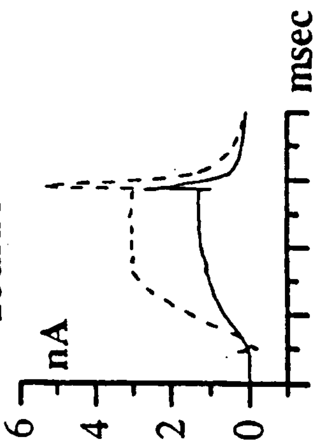


FIG. 3d
Flouride

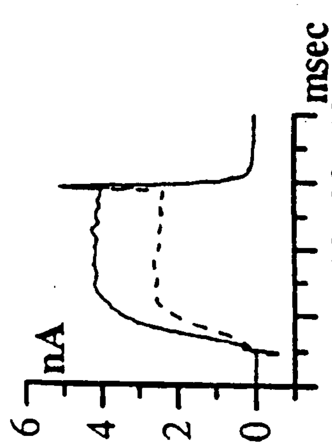


FIG. 3a
Bromide

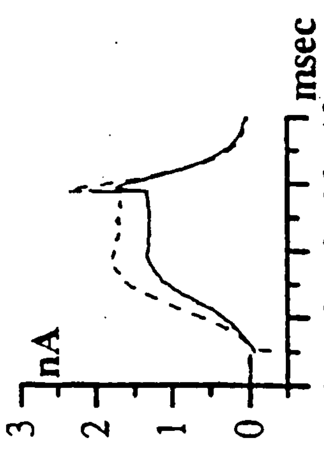
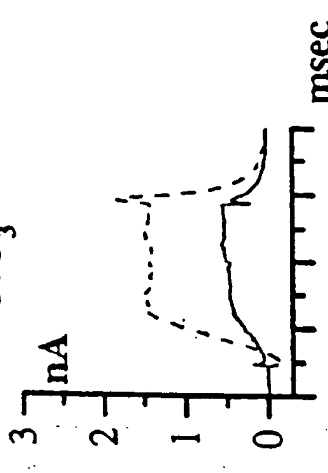


FIG. 3c
NO_3



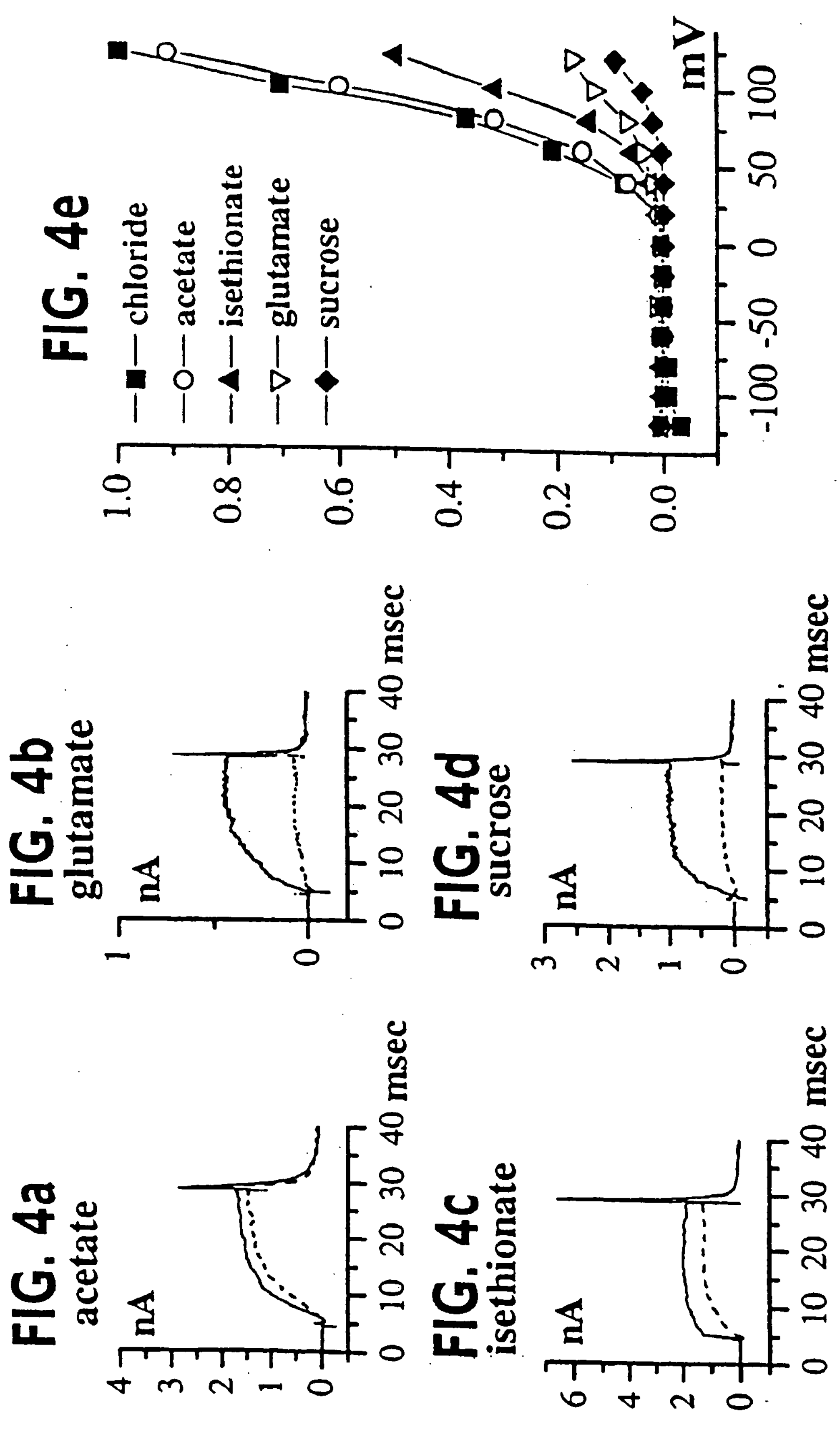


FIG. 4a
acetate
nA
msec
FIG. 4b
glutamate
nA
msec
FIG. 4c
isethionate
nA
msec
FIG. 4d
sucrose
nA
msec
FIG. 4e
chloride
acetate
isethionate
glutamate
sucrose
mV

FIG. 5a

control

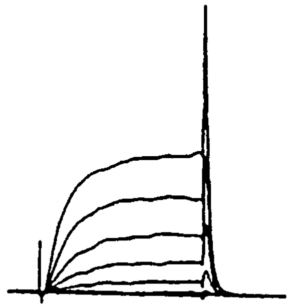


FIG. 5b

chlorotoxin

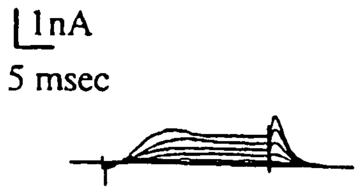


FIG. 5c

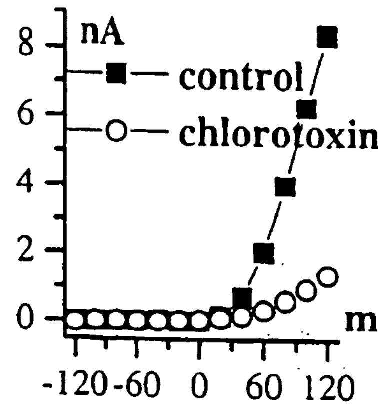


FIG. 5d

ctrl

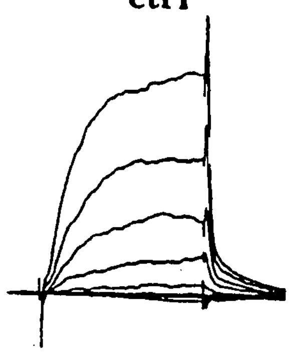


FIG. 5e

DIDS

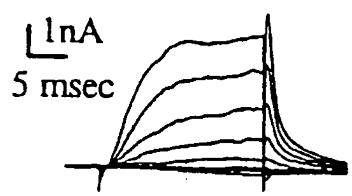


FIG. 5f

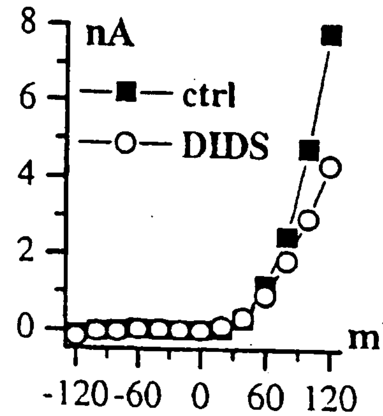


FIG. 5g

ctrl

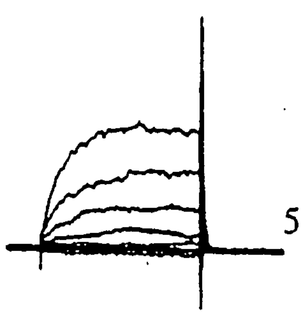


FIG. 5h

DNDS

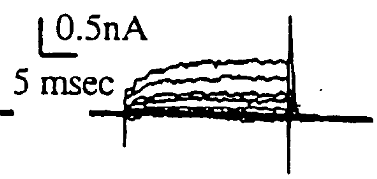


FIG. 5i

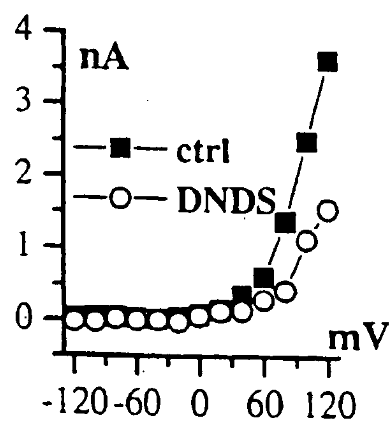


FIG. 6a

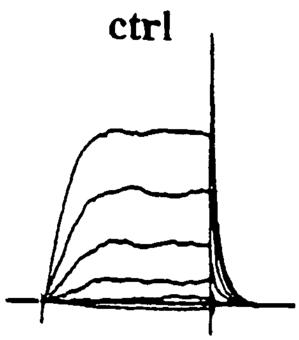


FIG. 6b

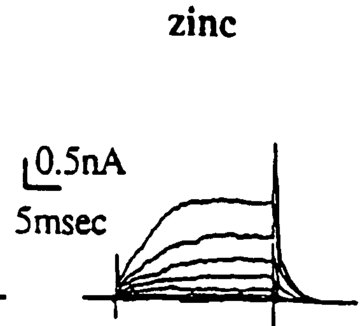


FIG. 6c

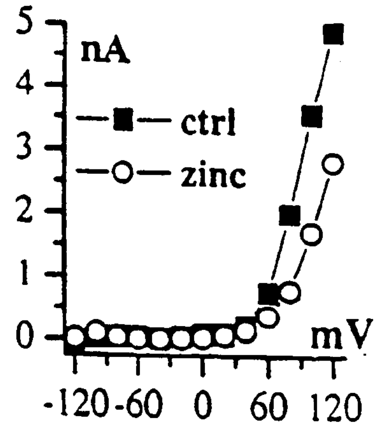


FIG. 6d

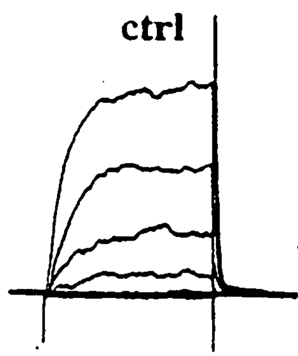


FIG. 6e

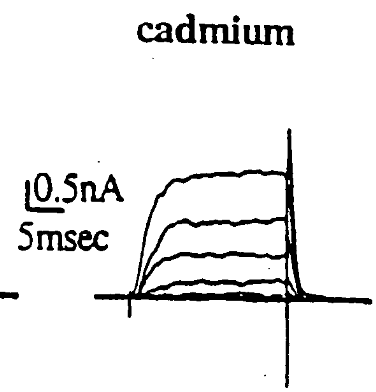


FIG. 6h

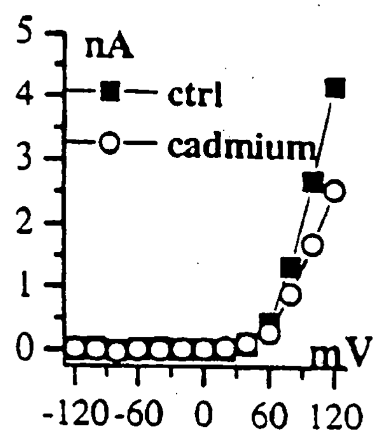


FIG. 6f

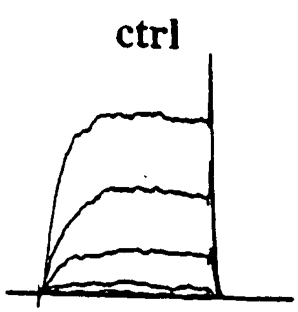


FIG. 6g

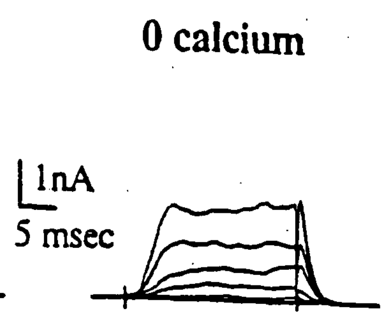


FIG. 6i

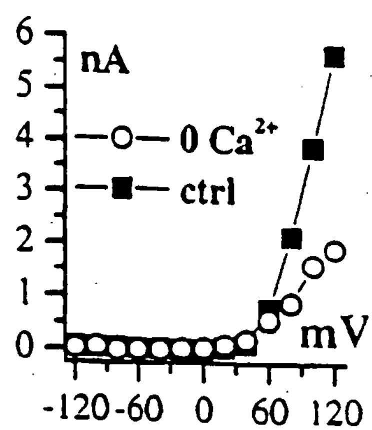
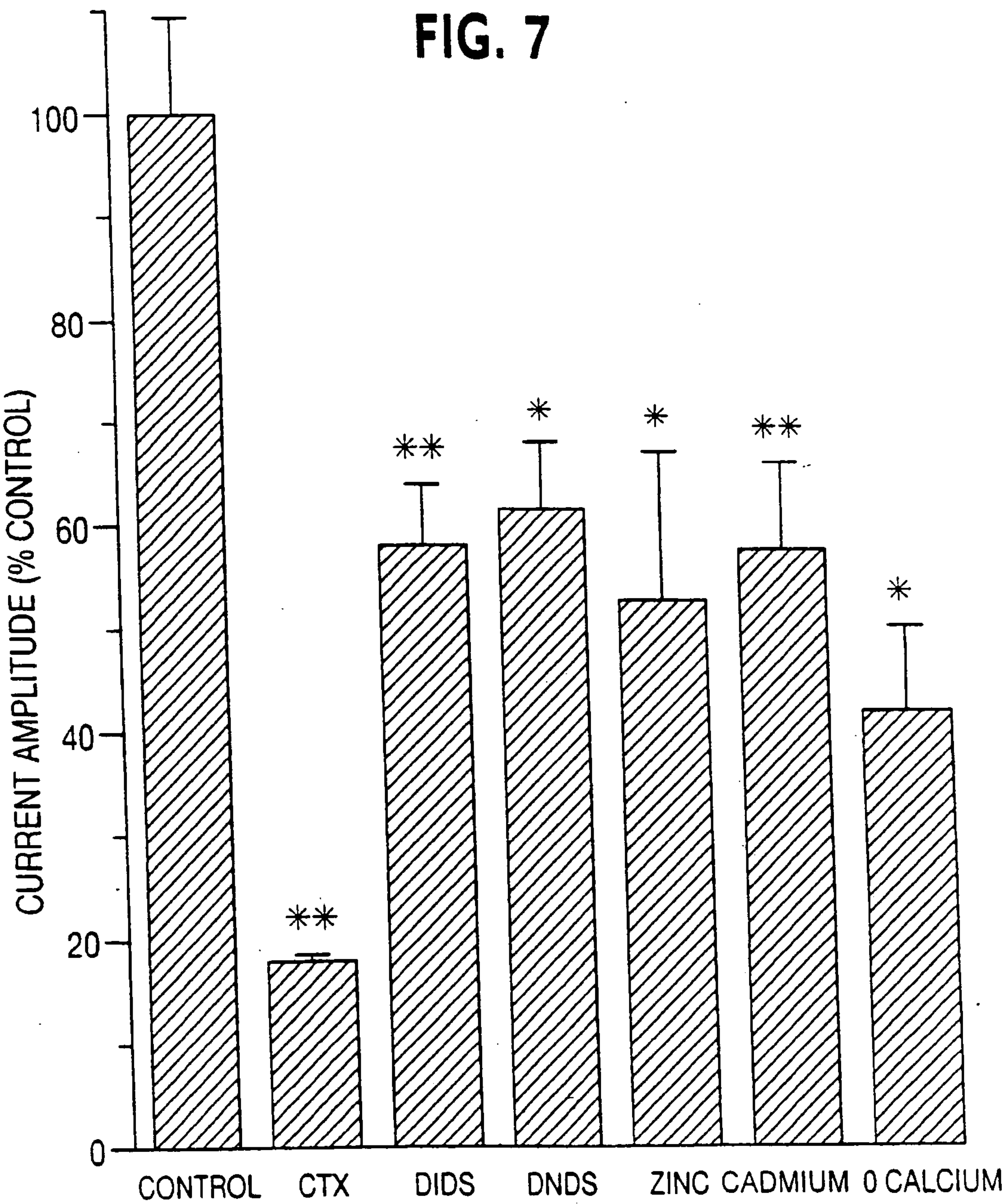
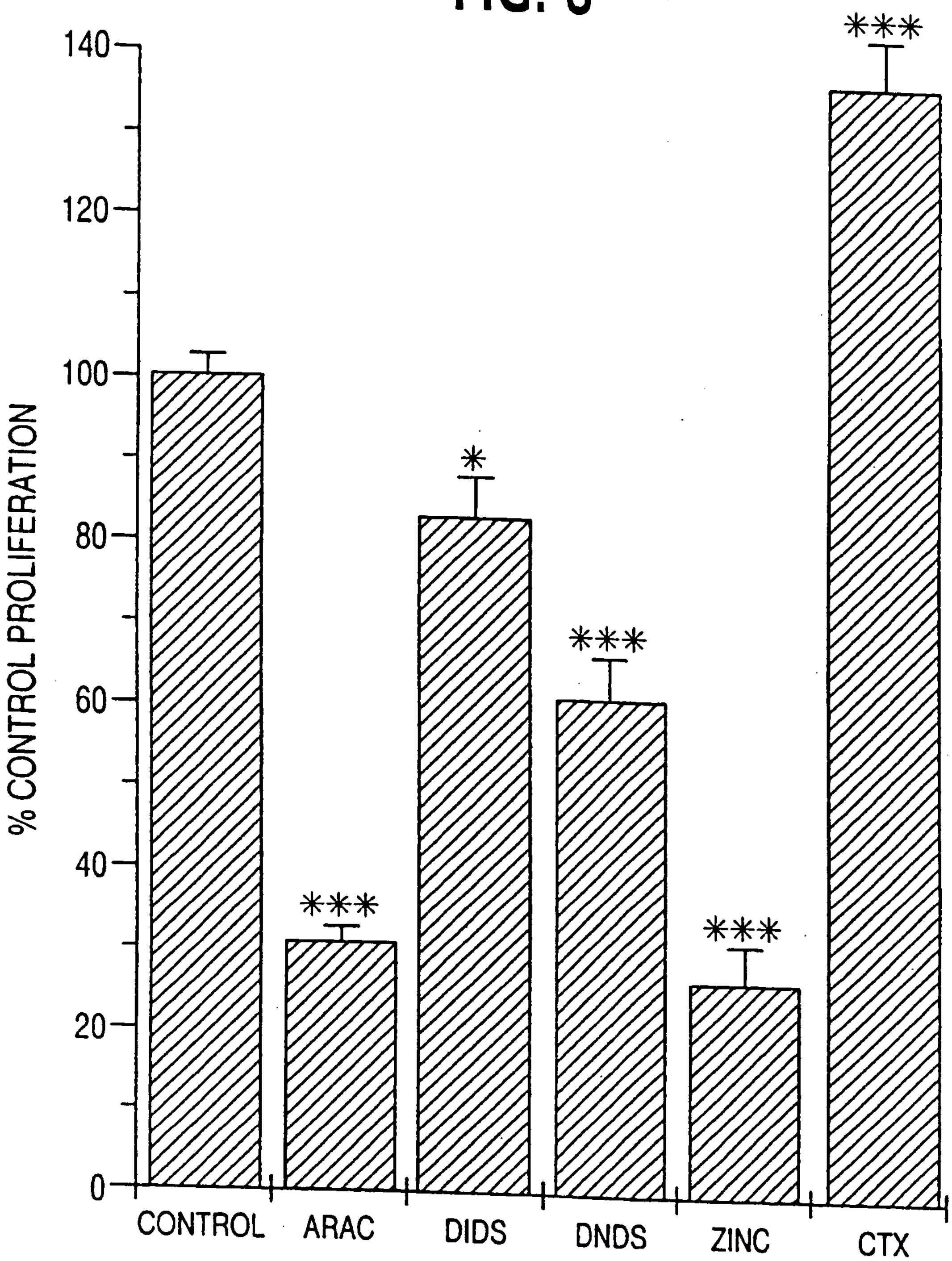


FIG. 7



CURRENT AMPLITUDE (% CONTROL)
100
80
60
40
20
0
**
**
*
*
**
*
CONTROL
CTX
DIDS
DNDS
ZINC
CADMIUM
0 CALCIUM

FIG. 8



140
120
100
80
60
40
20
0
% CONTROL PROLIFERATION
***
*
***
***
***
CONTROL
ARAC
DIDS
DNDS
ZINC
CTX

FIG. 9a

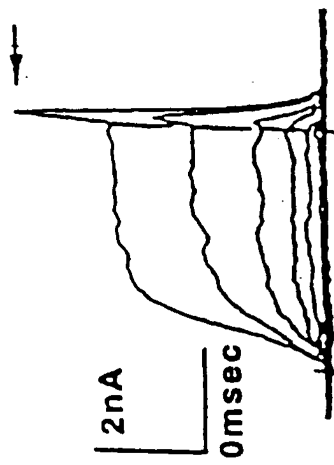


FIG. 9b

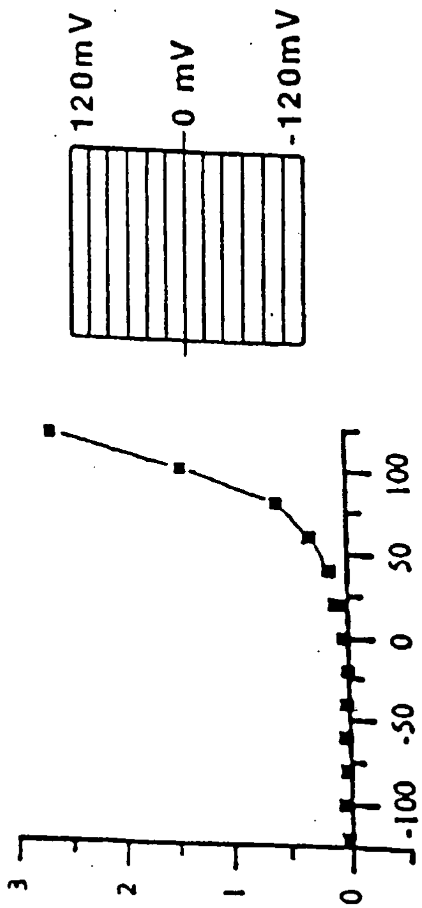


FIG. 9c

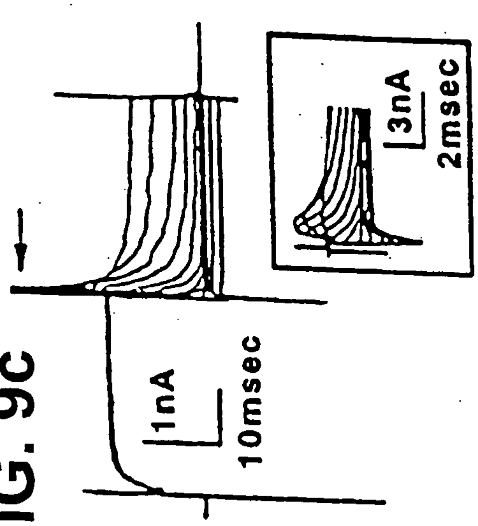


FIG. 9d

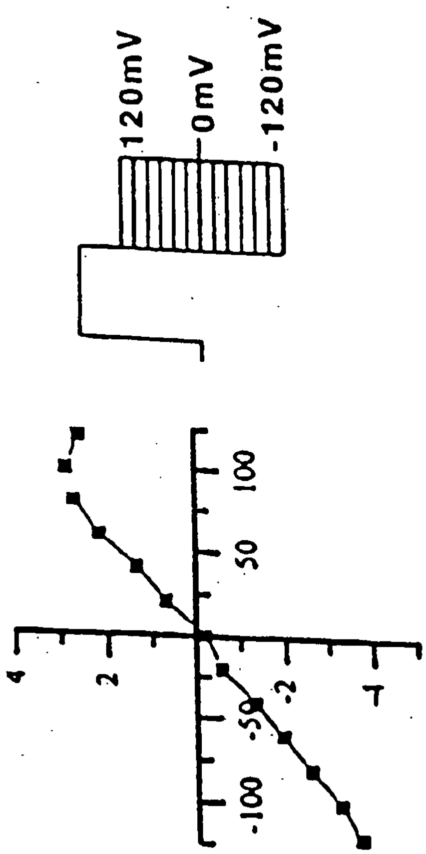


FIG. 10b

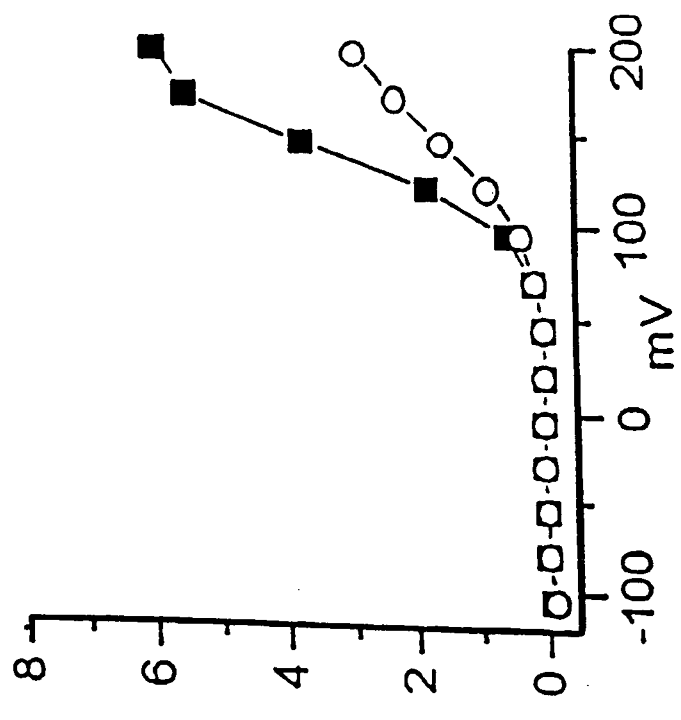


FIG. 10a

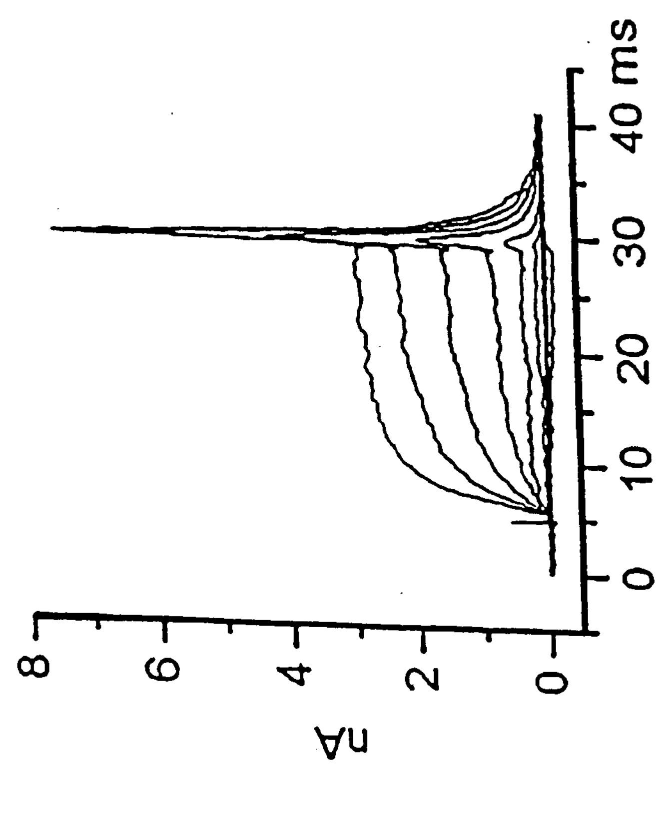


FIG. 11b

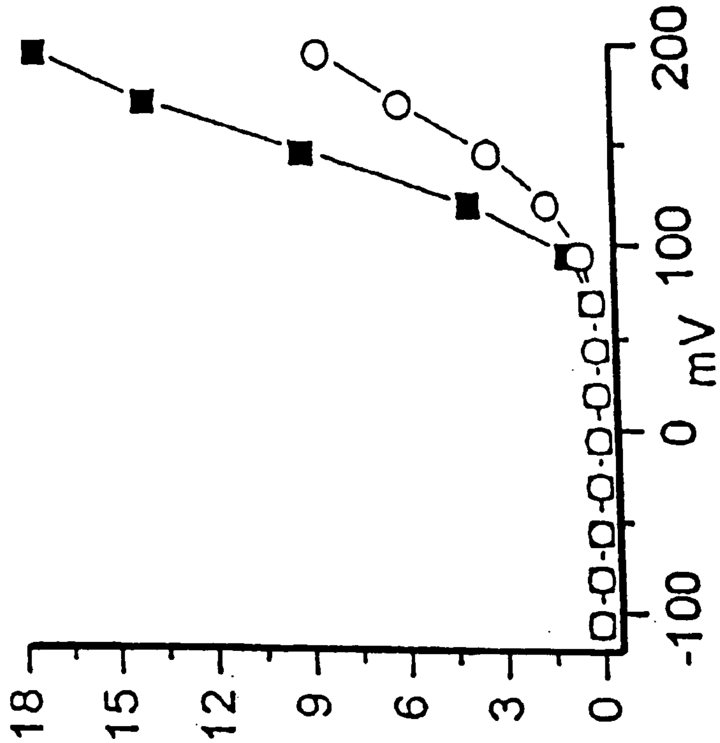


FIG. 11a

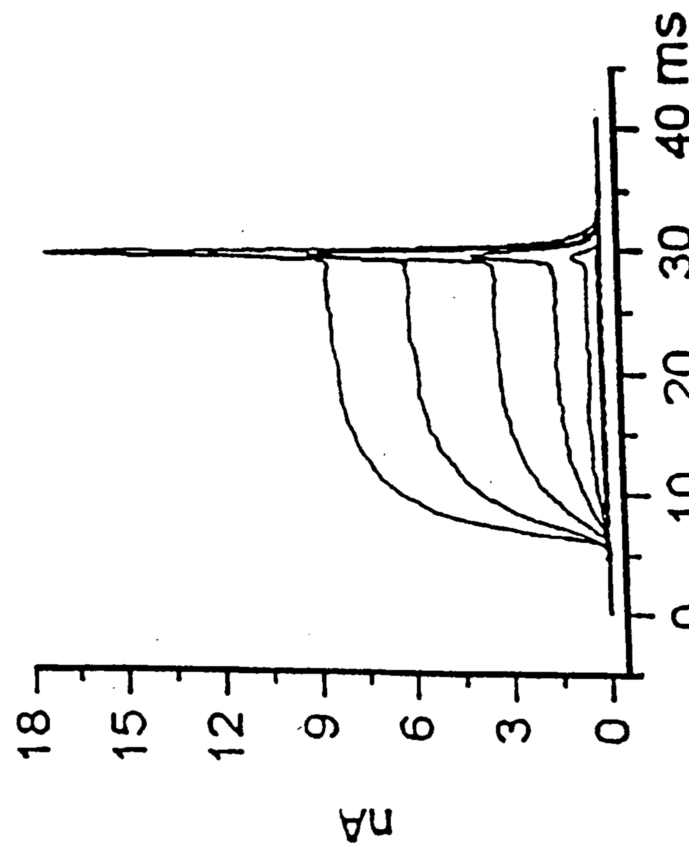


FIG. 12

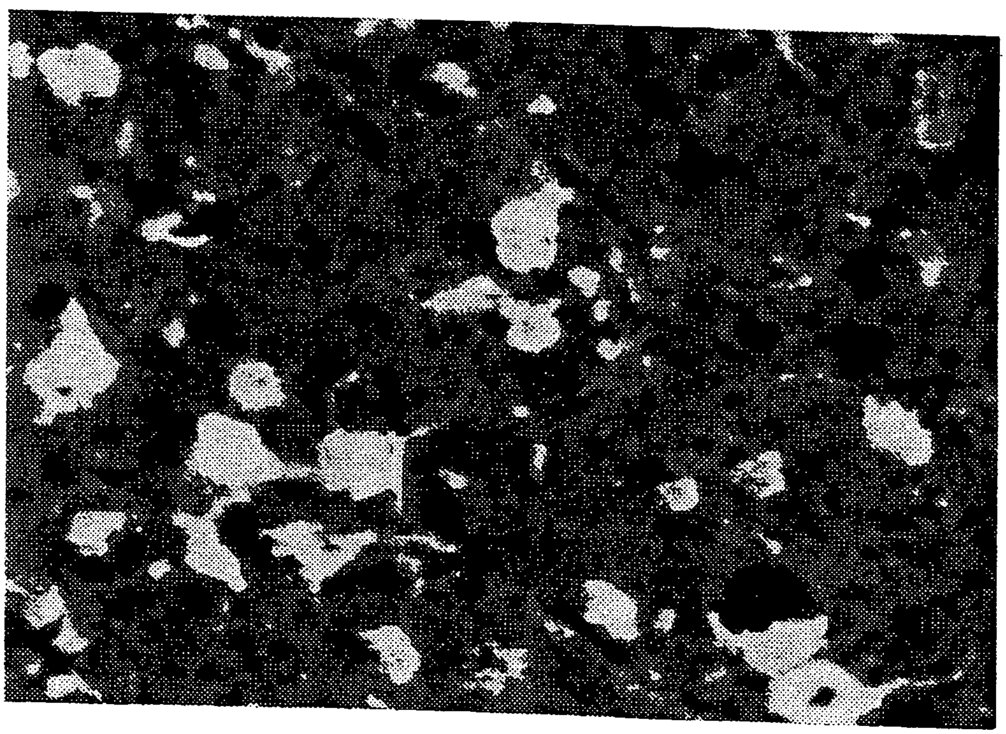


FIG. 13

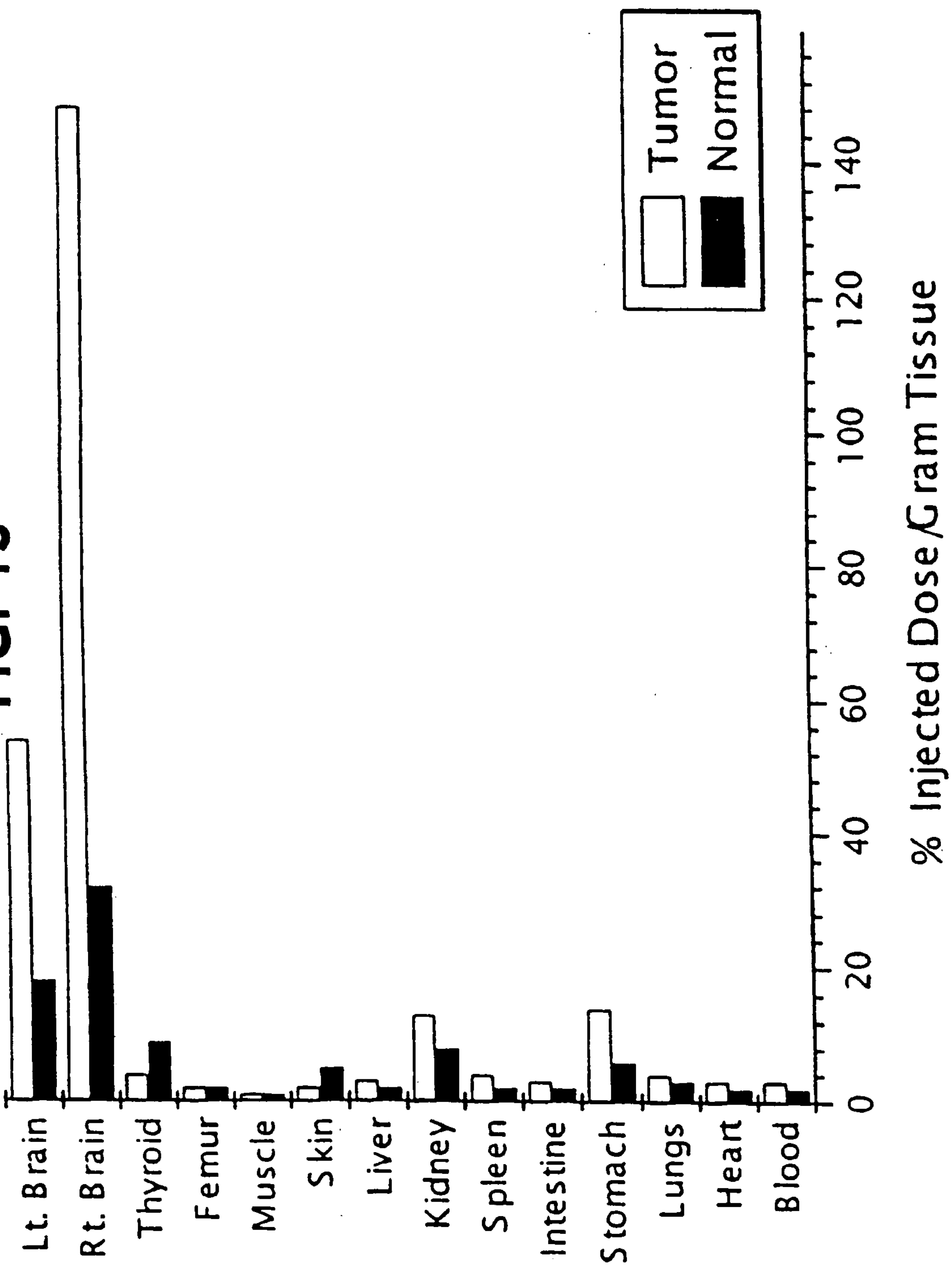

Tumor
Normal
% Injected Dose/Gram Tissue
0
20
40
60
80
100
120
140
Lt. Brain
Rt. Brain
Thyroid
Femur
Muscle
Skin
Liver
Kidney
Spleen
Intestine
Stomach
Lungs
Heart
Blood

FIG. 14a

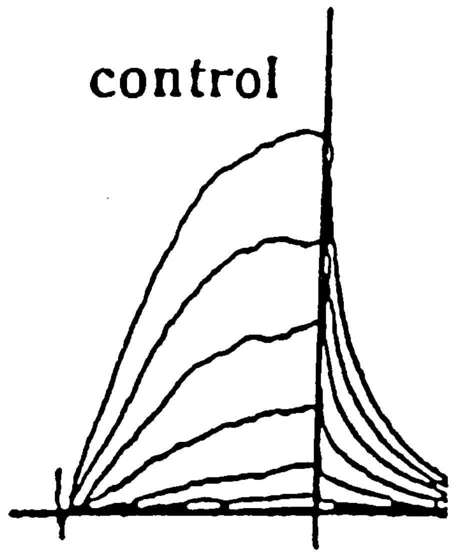
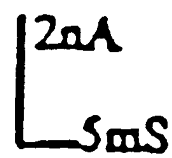


FIG. 14b



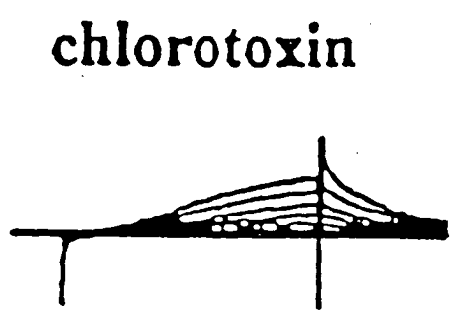
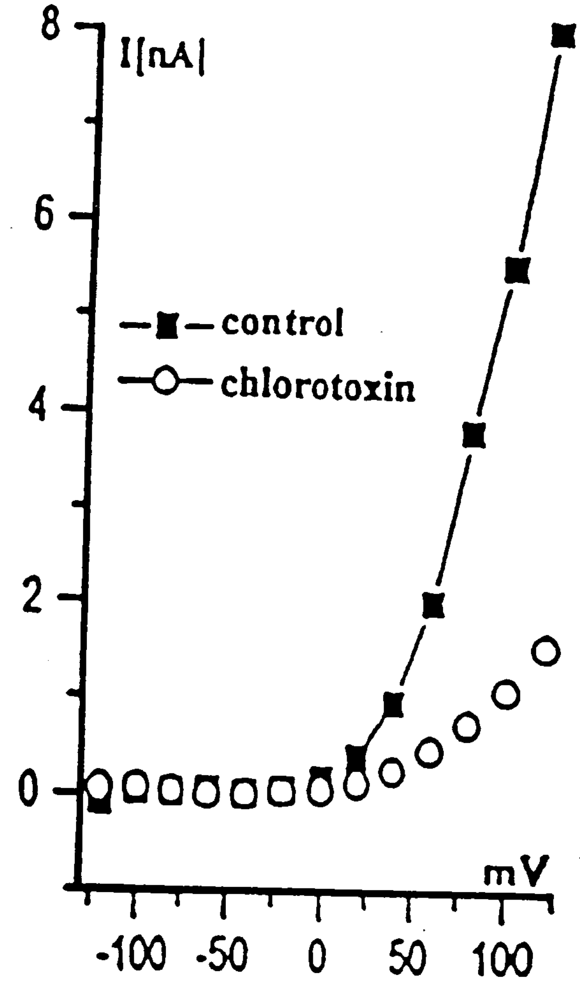


FIG. 14c



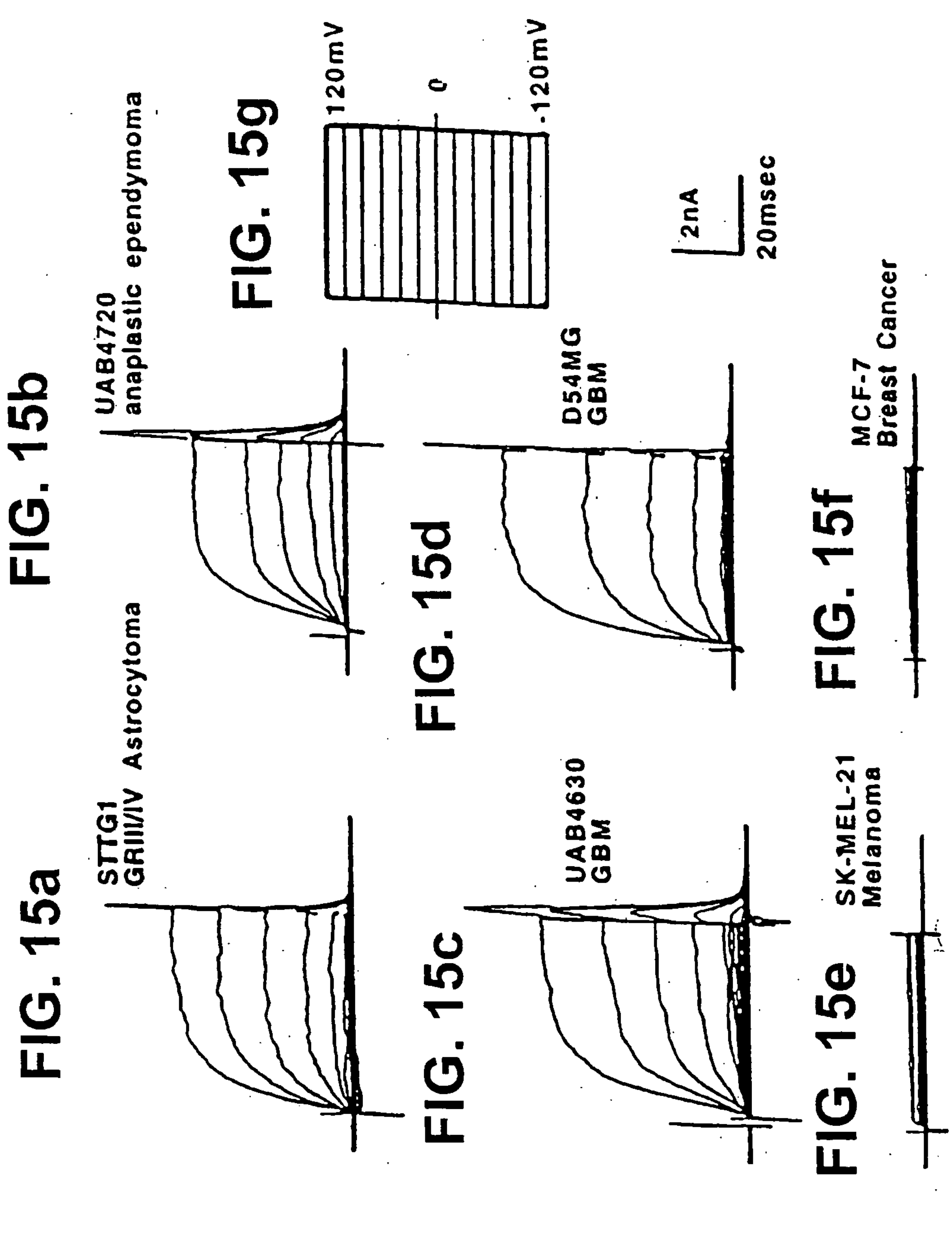


FIG. 15a

FIG. 15b

FIG. 15c

FIG. 15d

FIG. 15e

FIG. 15f

FIG. 15g

FIG. 16a
control

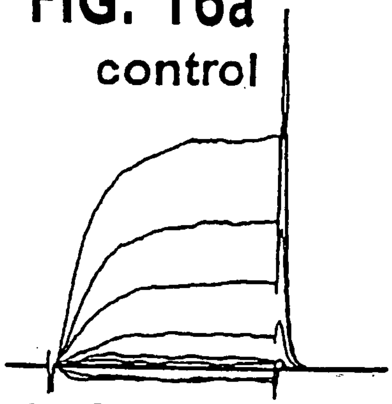


FIG. 16b
GST

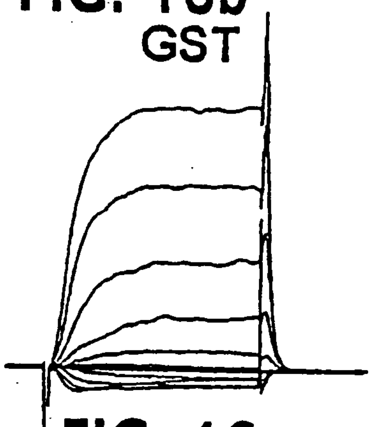


FIG. 16c
ctx-GST

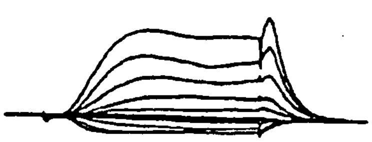


FIG. 16d

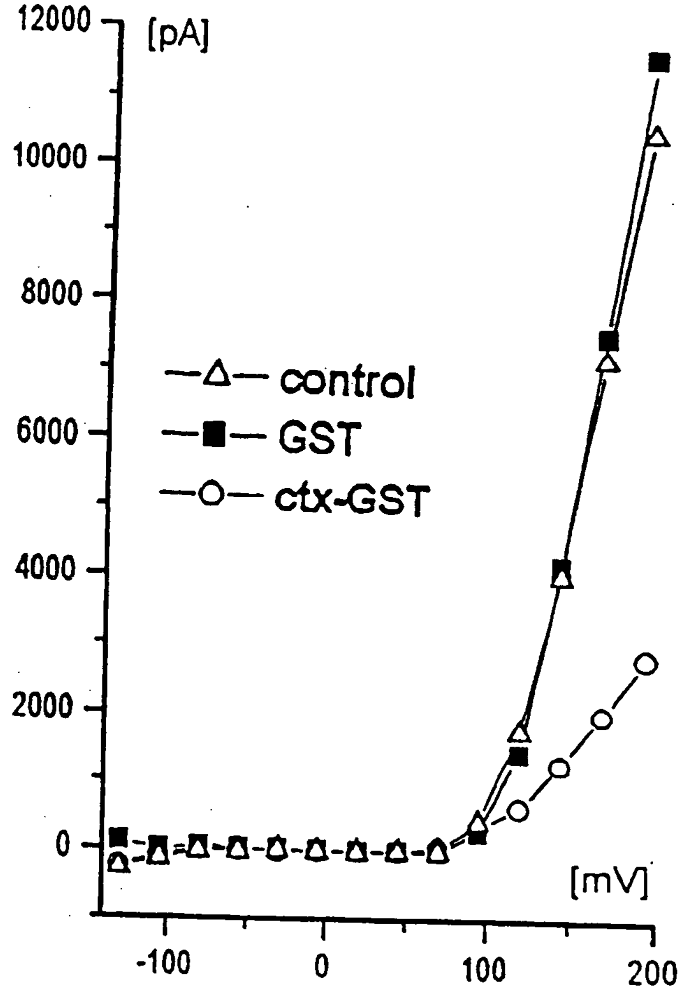
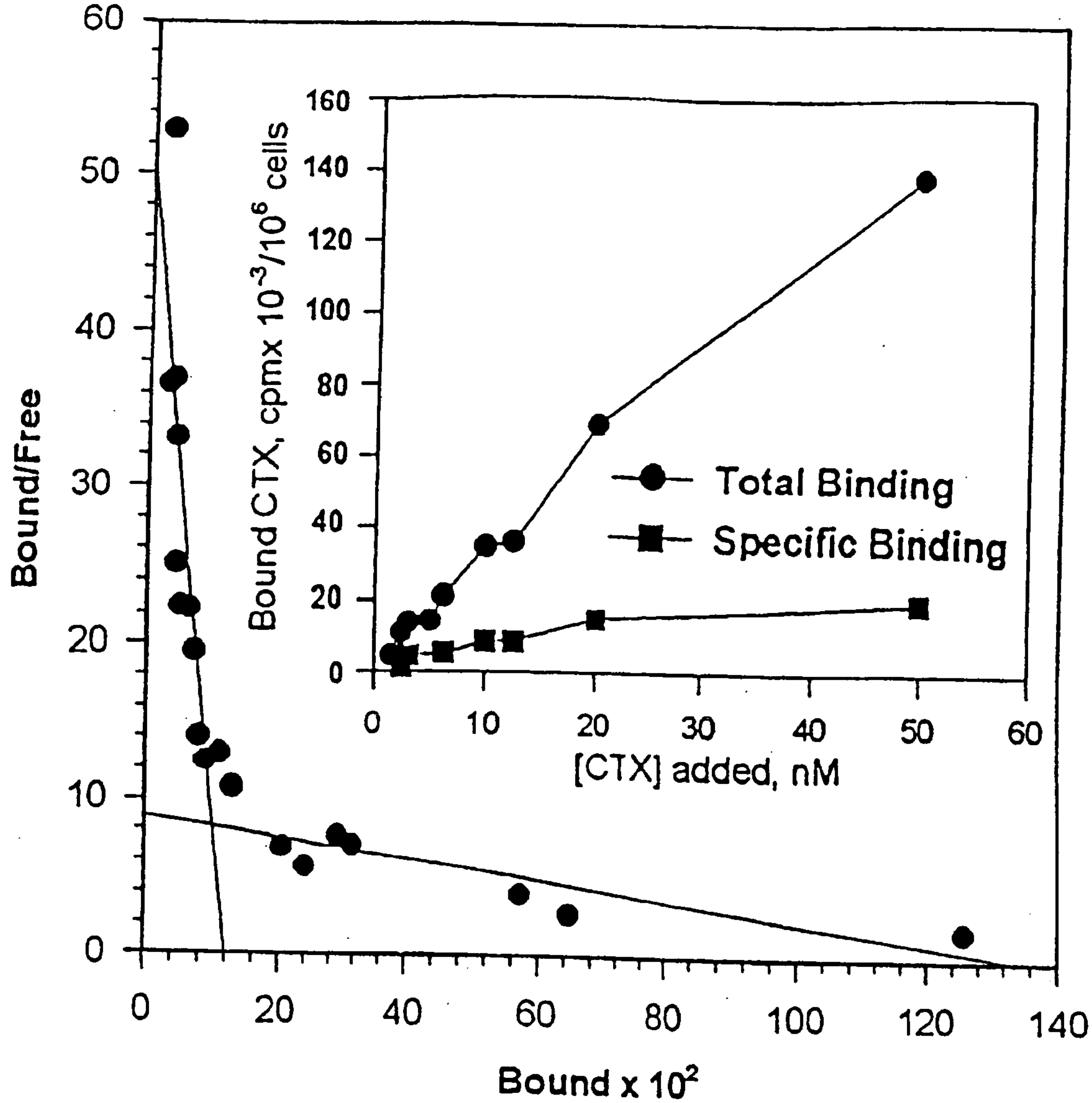


FIG. 17



Bound/Free
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
Bound x 10^2
Bound CTX, cpmx 10^{-3}/10^6 cells
160
140
120
100
80
60
40
20
0
10
30
50
[CTX] added, nM
Total Binding
Specific Binding

FIG. 18

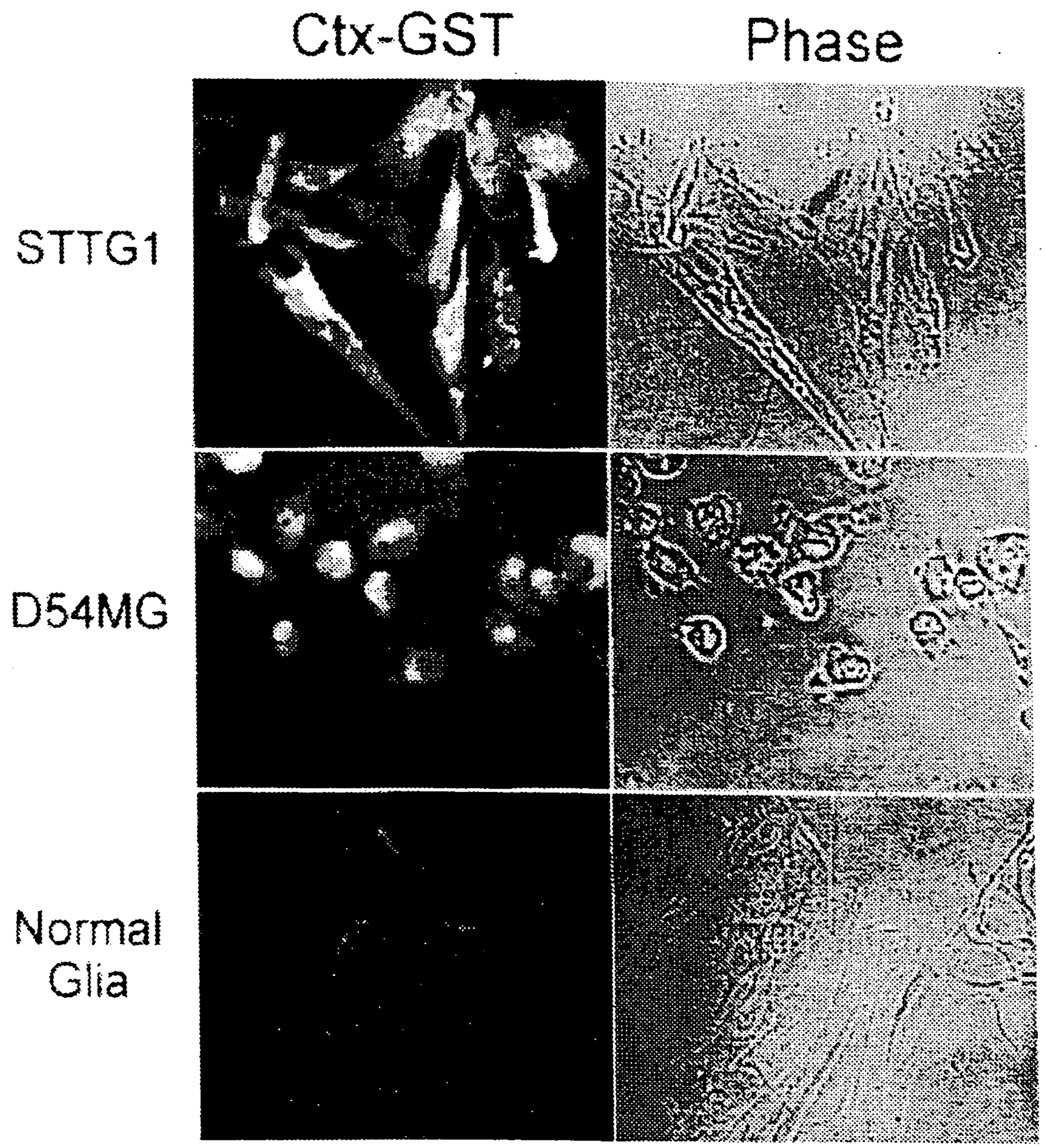


FIG. 19a

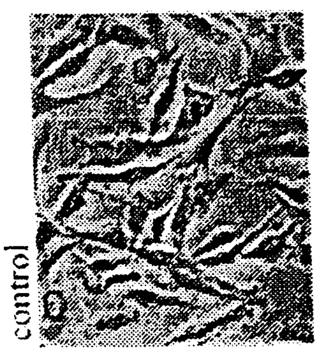


FIG. 19b

FIG. 19c

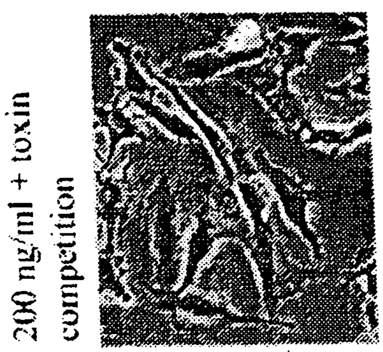


FIG. 19d

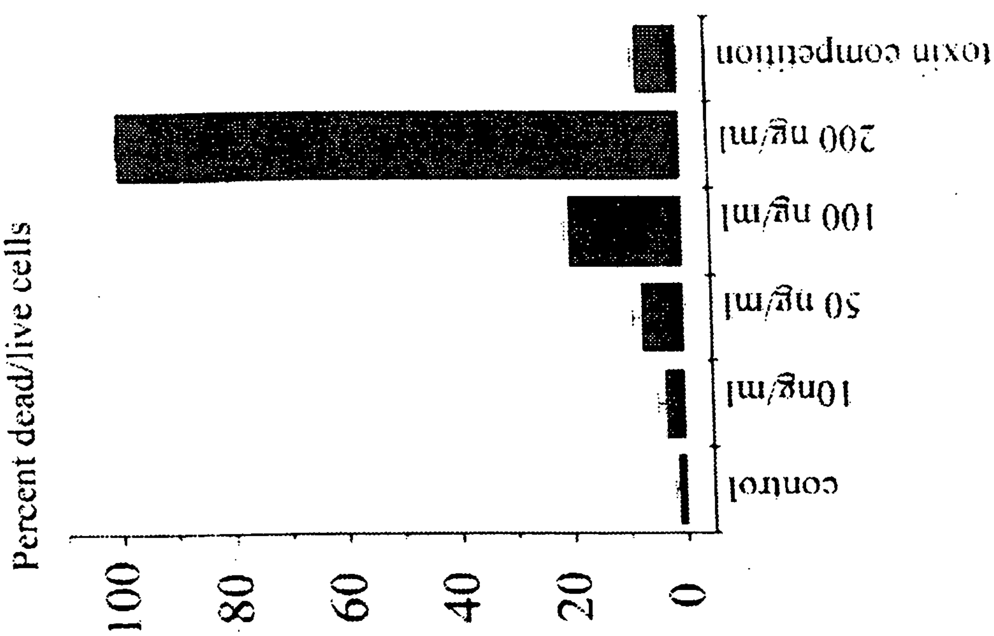
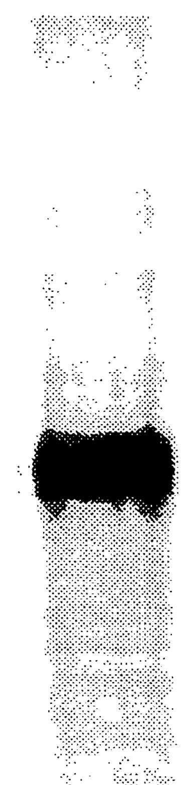


FIG. 20

Purification: ClC5 Ctx

Probe: ClC5



METHOD OF DIAGNOSING AND TREATING GLIOMAS

This is a divisional which claims benefit of priority of provisional application U.S. Ser. No. 60/009,283 filed Dec. 27, 1995 of application Ser. No. 08/774,154 filed on Dec. 26, 1996 now U.S. Pat. No. 5,905,027.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the fields of cell physiology, neurology and neuro-oncology. More specifically, the present invention relates to a novel method of diagnosing and treating gliomas and meningiomas.

2. Description of the Related Art

Glial cells comprise a large proportion of the total cell population in the CNS. Unlike neurons, glial cells retain the ability to proliferate postnatally, and some glial cells still proliferate in the adult or aged brain. Uncontrolled glial proliferation can lead to aggressive primary intracranial tumors, the vast majority of which are astrocytomas, and therefore, of glial origin. Tumors of astrocytic origin vary widely in morphology and behavior, and, according to the 1993 WHO classification schema, can be separated into three subsets. Astrocytomas, the lowest grade tumors, are generally well-differentiated and tend to grow slowly. Anaplastic astrocytomas are characterized by increased cellularity, nuclear pleomorphism, and increased mitotic activity. They are intermediate grade tumors and show a tendency to progress to a more aggressive grade. Glioblastomas are considered the most aggressive, with poorly differentiated cells, vascular proliferation, and necrosis. Due to the common morphological heterogeneity of cells within a single tumor, such classification is not clear-cut and is somewhat unsatisfactory. The term "astrocyte-derived tumors" as used herein refers to astrocytomas. Meningiomas are tumor originating in the meninges.

Significant progress has been made in identifying physiologically important growth factors, receptors, and signal transduction pathways that control normal and malignant cell proliferation. It is now commonly accepted that growth factor binding leads to activation of oncogenes such as the ras/raf pathway, and ras in turn regulates gene expression through at least two mitogen-activated protein kinases. Interestingly, the ras/raf pathway is in crosstalk with the cAMP signaling cascade which is activated by numerous hormones and neurotransmitters.

Recent studies suggest that ion channels may function in regulating a cell's proliferative ability. For example, mitogen-stimulated lymphocytes show an upregulation in the expression of a high conductance potassium channel (15). In murine fibroblasts, activation of the ras/raf signaling cascade induces expression of a Ca^{2+}-activated K^+ channel that appears to be essential in the cells' proliferative response (17). The idea that ion channel expression may be necessary for cell cycle progression is also supported by observations that pharmacological blockade of ion channels can inhibit cell proliferation. This has been demonstrated in a number of cell types including melanoma (28), breast cancer cells (41), brown fat cells (30), and also in several glial cell types such as Schwann cells (5), retinal glial cells (32) and astrocytes (29).

Untransformed glial cells from which glial tumors may originate have been extensively characterized electrophysiologically (37). Surprisingly, they appear to be liberally endowed with voltage- and ligand-activated ion channels for Na^+, K^+, Ca^{2+} and possibly Cl^- ions. It is generally assumed that these ion channels perform homeostatic roles in the brain and may facilitate maintainance of K^+ and possibly Na^+ and Cl^- ion concentrations in the extracellular space. In contrast to the numerous reports on ion channel expression and activity in nonneoplastic glial cells, electrophysiological properties of astrocytoma cells and the potential role of ion channels in growth control of astrocytomas remain largely unexplored. Inwardly rectifying K^+ currents have been demonstrated in several established astrocytoma cell lines (4).

Gliomas cells are a very heterogeneous cell population that lack common antigens. Consequently, the prior art is deficient in the lack of effective means of identifying and treating malignant gliomas. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

The present invention describes the expression of a chloride conductance with unique properties that selectively characterizes tumor-derived cells of glial origin. In the present invention, whole-cell patch-clamp techniques were used to characterize the biophysical and pharmacological properties of chloride channels in primary cultures and acutely isolated cells from biopsies of human astrocytomas and established cell lines. In all preparations, the expression of time-dependent and voltage-dependent outwardly rectifying currents was observed. These currents are sensitive to several Cl^- channel blockers including chlorotoxin, a component of scorpion venom and also allow other anions to permeate. This chloride conductance is involved in the growth control of astrocytoma cells.

Expression of voltage activated ion channels was determined in primary cultures from 7 freshly resected human primary brain tumors and in a 7 established human astrocytoma cell lines. Astrocytoma cells consistently expressed voltage-dependent outwardly-rectifying currents. Currents activated at potentials greater than 45 mV and showed outward transients upon termination of voltage steps. Currents reversed at the Cl^- equilibrium potential, suggesting that they were largely carried by Cl^- ions. Altering $[K^+]_o$ or $[Na^+]_o$ did not alter currents; neither did replacement of $[K^+]_i$ by Cs^+ or $[Na^+]_i$ by NMDG. Anion substitution experiments suggest the following permeability sequence, determined from shifts in tail current reversal potential: $I^- > NO_3^- > Br^- > Cl^- >$ acetate $>$ isethionate $> F^- >$ glutamate. Currents were sensitive to the Cl^- channel blockers chlorotoxin, DIDS, and DNDS, with chlorotoxin being most effective, yielding >80% block at 590 nM. DIDS (100 μM) and DNDS (100 μM) reduced currents by 33.5% and 38.2% respectively. Currents were also sensitive to zinc (100 μM, 47% block) and cadmium (25 mM, 42% block). Reducing $[Ca^{2+}]_o$ decreased outward currents by 58% and almost completely eliminated transients, suggesting that Cl^- currents are Ca^{2+}-dependent. Cl^- channel block resulted in altered cell proliferation as determined by ^{3}H-thymidine incorporation, indicating that these channels are involved in astrocytoma growth control.

It is an object of the present invention to demonstrate that glial-derived tumor cells express a unique voltage-dependent Cl^- channel which is not found in non-glial tumors, such as melanoma or breast carcinoma, nor in untransformed glial cells.

It is another object of the present invention to show that expression of this unique Cl^- channel plays a role in the cells' abnormal proliferative state.

It is yet another object of the present invention to demonstrate the sensitivity of glioma Cl^- channels to chlorotoxin.

It is still another object of the present invention to provide a monoclonal antibody which specifically binds to glial-derived or meningioma-derived tumor cells.

It is still another object of the present invention to demonstrate that glioma cells can be targeted and/or eliminated by a recombinant chlorotoxin fused to a cytotoxic protein.

It is still another object of the present invention to provide a method to screen for malignant gliomas.

It is still yet another object of the present invention to provide a method of treating malignant gliomas, including glioblastoma multiforme and astrocytomas.

Thus, in accordance with the aforementioned objects, in one embodiment of the present invention, there is provided an antibody which specifically recognizes an antigen in chloride channels of glial-derived tumor cells.

In another embodiment of the present invention, there is provided a pharmaceutical composition, comprising a ligand which binds specifically to glial-derived or meningioma-derived tumor cells and a pharmaceutically acceptable carrier.

In yet another embodiment of the present invention, there is provided a method of differentiating glial-derived or meningioma-derived neoplastic tumor tissue from non-neoplastic tissue, comprising the steps of: contacting a tissue of interest with an antibody that specifically recognizes an antigen in chloride channels of glial-derived tumor cells; and measuring the level of binding of the antibody, wherein a high level of binding is indicative that the tissue is neoplastic.

In still yet another embodiment of the present invention, there is provided a fusion protein, said protein comprised of: a ligand that specifically recognizes an antigen in chloride channels of glial-derived tumor fused to a cytotoxic moiety.

In still yet another embodiment of the present invention, there is provided a method of treating an individual having a glioma or meningioma, comprising the step of administering to said individual a pharmacologically effective dose of a composition of the present invention.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

FIGS. **1**(*a–d*) shows the whole-cell voltage-clamp recordings obtained from a representative human astrocytoma cell from cell line STTG1 and from a primary cultured astrocytoma cell (UAB4630). Cells were stepped to test potentials between −105 mV and 195 mV in 25 mV increments from a holding potential of 0 mV (inset). Cells showed large transients upon termination of voltage steps (star, A, C). Potential >45 mV resulted in fast-activating, non-inactivating outwardly rectifying currents (B, D).

FIGS. **2**(*a–b*) shows that in order to determine the ion species that was carrying the outward current, the reversal

potential of tail currents was analyzed. Cells were held at 0 mV, pulsed to 180 mV, and then pulsed in −20 mV steps from +120 mV to −120 mV (A, inset). Plotting tail current amplitudes (A, inset) as a function of voltage showed a reversal potential of 8 mV (B) in this cell.

FIGS. **3**(*a–e*) shows the whole-cell leak subtracted current responses of STTG1 cells in response to a single 145 mV voltage step prior to and following substitution of extracellular Cl^- with (125 mM) Br^-(A), I^-(B), NO_3^-(C), or F^-(D). Dashed lines represent control current with standard external solution and straight lines represent current with replacement solution. E) peak current-voltage relations obtained as in FIG. **1**, with current normalized to that obtained with standard NaCl-rich external solution.

FIGS. **4**(*a–e*) shows as in FIG. **3**, whole-cell leak subtracted current responses of STTG1 cells prior to and following substitution of extracellular Cl^- with (125 mM) acetate (A), glutamate (B), isethionate (C), or sucrose (D). As above, dashed lines represent control current with standard bath solution and straight lines represent current after replacement. E) peak current-voltage relations, normalized to current in presence of 125 mM Cl^- as above.

FIGS. **5**(*a–i*) shows the effect of bath application of chlorotoxin, DIDS (4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid) and DNDS (4,4'-Dinitrostilbene-2,2'-disulfonic acid) on outward currents in STTG1 astrocytoma cells in response to test voltage pulses from −105 to +195 in 25 mV increments. Whole-cell currents are shown prior to (A) and following (B) bath application of 590 nM chlorotoxin. Chlorotoxin decreased outward currents by 81%. C) I-V relation of peak current amplitude as a function of applied voltage. Currents are also shown before and after application of 100 μM DIDS (D, E) and 100 μM DNDS (G, H). Current-voltage relations from those examples are shown in parts (F) and (I). The size of the outward current is reduced by DIDS at all potentials (33.5%±12.9 (n=5)). Similar to the effect of DIDS, DNDS caused a decrease in current amplitude at all potentials by 38.2%±13.3 (n=4).

FIGS. **6**(*a–i*) shows the effect of the zinc, cadmium, and calcium on outward currents. Bath application of 100 μM zinc led to a 47%±25.9 (n=3) decrease in peak currents (FIGS. **6**, A–C), and 25 μM cadmium led to a 42%±18.5 (n=5) decrease (FIGS. **6**, D–E). In bath solution with zero Ca^{2+}/5 mM EGTA, currents were decreased to 40% of that in control solution, containing 1 mM Ca^{2+} (FIGS. **6**, F–H).

FIG. **7** shows the comparison of the effects of channel blockers on outward currents. Effects are expressed as percent of normalized to current amplitude obtained in standard NaCl-rich external solution for pooling of experimental data. Error bars reflect SEM.

FIG. **8** shows the effects of the anti-mitotic agent Ara-C (10 μM), DIDS (200 μM), DNDS (200 μM), Zinc (200 μM), and chlorotoxin (600 nM) on astrocytoma proliferation, assessed as ^{3}H-thymidine incorporation following 24 hour incubation with the agent of interest. Mean effects (expressed as cpm/μg protein, error bars=SD) were plotted for each agent tested in at least 6 experiments each (see text for details). As expected, incubation in the anti-mitotic agent Ara-C led to a 70% decrease in proliferation (SD=1.3309, N=17). The chloride channel blockers DIDS, DNDS, and zinc decreased proliferation by 16.4% (SD=20.0, N=16), 38.2% (SD=13.1, N=8), and 72.6% (SD=12.4, N=7), respectively. By contrast, incubation in chlorotoxin led to a 37.8% increase in proliferation compared to control (SD=5.7, N=8). Error bars reflect SEM.

FIGS. **9**(*a–d*) shows the whole-cell voltage-clamp recordings obtained from representative human astrocytoma cell

(STTG1). Cells were stepped to test potentials between −120 mV and 120 mV in 20 mV increments from a holding potential of 0 mV. Cells showed large tail currents upon termination of voltage steps (arrow, A). Potential >0 mV resulted in fast-activating, non-inactivating outwardly rectifying currents (B). In order to determine the ion species that was carrying the outward current, the reversal potential of tail currents was analyzed. Cells were held at 0 mV, pulsed to 200 mV, and then pulsed in −20 mV increments from +120 mV to −120 mV (C). Plotting tail current amplitudes (C, inset) as a function of voltage showed a reversal potential of 0 mV (D).

FIGS. **10**(*a–b*) shows the recordings from a glioma cell in biopsy tissue from a GBM in response to 13 depolarizing voltage steps ranging from −105 to 195 mV.

FIGS. **11**(*a–b*) shows the recordings from a xenografted D54MG glioma cell recorded in acute slices in response to depolarizing voltage steps ranging from −105 to 195 mV.

FIG. **12** shows the staining of a 200 μm section through a glioma induced experimentally in a scid mouse. Fluorescent cells are identified by staining with Ctx-GSt recognized by an anti-GST antibody conjugated to FITC. 20× magnification.

FIG. **13** shows the biodistribution of Chlorotoxin binding sites as determined by injection of ^{125}I-Ctx into a scid mouse bearing an experimental tumor.

FIGS. **14**(*a–c*) shows the bath application of chlorotoxin (590 nM) inhibits outward currents in STTG1 cell line.

FIGS. **15**(*a–f*) shows the representative whole-cell leak-subtracted currents from human tumor cell lines. Astrocytoma and glioblastoma cell lines were dominated by outwardly-rectifying voltage-activated chloride currents, whereas these currents were absent in cells from breast tumor and melanoma.

FIGS. **16**(*a–d*) shows a Ctx-GST is an effective blocker of glioma Cl^- channels. 600 nM Ctx-GST was applied with bath perfusion and resulted in ~70% reduction in Cl^- currents; GST alone was ineffective.

FIG. **17** shows that binding of ^{125}[I]-CTX to D54MG glioma cells. ^{125}[I]-CTX was added in duplicate in 400 mL with or without a 100-fold molar excess of unlabeled Ctx from the same source. After 60 min at room temperature, cell monolayers were rinsed 3 times with PBS and cells were harvested for assessment of cell-associated radioactivity. Four wells in each plate were harvested with trypsin-EDTA and cell number was established by trypan blue exclusion. (Note that not all data points used for the Scatchard analysis were plotted in the inset).

FIG. **18** shows the immunohistochemical staining of two glioma cell lines as compared to normal human glia. Cells were labeled with the recombinant chlorotoxin-GST fusion protein (Ctx-GST) and binding of Ctx-GST was visualized by an anti-GST antibody coupled to FITC.

FIGS. **19**(*a–q*) show the glioma cells were exposed to 600 nM Ctx-GST followed by a mouse aGST and a goat anti-mouse antibody conjugated to saporin. Cell death increased with increasing saporin concentrations (right) and could be largely prevented by pre-treatment of cultures with 6 μM native chlorotoxin (bottom left).

FIG. **20** shows a western blot of U54MG membranes after immunpercipitation with either ClC5 antibodies or chlorotoxin (Ctx). Blots were probed with ClC5.

DETAILED DESCRIPTION OF THE INVENTION

Glioma cells, e.g. primary brain tumors derived from glial cells, express a unique membrane protein which constitutes a Cl^- ion channel termed herein Glioma Chloride Channel (GCC). In the brain, GCC is specific to gliomas and meningiomas and is not present in other cells. GCC was identified in 24/24 glioma patient biopsies, in 7/7 astrocytoma/glioblastoma cell lines and in 4/4 meningioma biopsies. GCC expression correlates with pathological tumor grade. GCC expression is preserved in intracranial xenograft tumors in scid mice, which provide an excellent animal model for the disease. GCC binds chlorotoxin, a 36 amino acid peptide, with high affinity and selectivity. Binding is preserved in both synthetic and recombinant form of chlorotoxin, and also if the molecule is altered in ways to carry fluorescent or cytotoxic moieties.

GCC is a specific marker and useful target for gliomas and meningiomas and can be used for diagnostic and therapeutic purposes. GCC can be targeted by antibodies to the protein and/or by molecules that bind to it. Specifically, chlorotoxin and chlorotoxin-like molecules (fusion proteins) can be used to specifically direct molecules bound on it to gliomas and meningiomas. These molecules include but are not limited to: ^{125}I, ^{131}I, fluorescent moieties, cytotoxic moieties including but not limited to ricin, saporin, pseudonomas exotoxin. Binding of chlorotoxin-like molecules or antibodies to GCC can be utilized to diagnose gliomas and meningiomas. Non-invasive strategies can be devised to utilize GCC expression for diagnostic purposes. For example, binding of ^{131}I-Ctx to GCC can be visualized by PET scan.

Chlorotoxin inhibits GCC currents in a dose-dependent manner, with an apparent IC_{50} of 950 nM. To assure that these effects were not caused by impurities in the venom, a recombinant toxin was also generated (in *E. coli*) after fusion to glutathione-S-transferase. The recombinant Ctx-GST fusion protein was even more effective in blocking GCC currents (IC_{50}~540 nM), and an example of a whole-cell recording in the presence and absence of Ctx-GST and GST alone in a single cell is presented below. Chlorotoxin binds directly to GCC channels, as is the case in rat colonic epithelial cells (DeBin and Strichartz, 1991), where one single Ctx molecule is sufficient for channel block. However, it is possible that Ctx binds to a binding protein or receptor, and that subsequent changes in GCC currents are mediated indirectly, e.g. through G-proteins or second messengers.

The present invention is directed to novel methods of identifying, targeting and effectively suppressing the growth of glial-derived neoplastic cells. In one embodiment, the present invention provides a pharmaceutical composition, comprising an ligand which binds specifically to glial-derived or meningioma-derived tumor cells and a pharmaceutically acceptable carrier. In one embodiment, the ligand is an antibody which recognizes an antigen that is a glioma or meningioma specific chloride channel. Alternatively, the ligand is a chlorotoxin-like compound and is radiolabeled.

The present invention is also directed to a method of differentiating glial-derived or meningioma-derived neoplastic tumor tissue from non-neoplastic tissue, comprising the steps of: contacting a tissue of interest with an antibody that specifically recognizes an antigen in chloride channels of glial-derived tumor cells; and measuring the level of binding of the antibody, wherein a high level of binding is indicative that the tissue is neoplastic. Preferably, the level of antibody binding indicative of neoplastic tissue is from about 30% to about 90% of cells positively binding the antibody.

The present invention is also directed to a method of differentiating glial-derived or meningioma-derived neo-

plastic tumor tissue from non-neoplastic tissue, comprising the steps of: contacting a tissue of interest with labeled chlorotoxin which binds specifically to glial derived neoplastic tumor tissue; and measuring the binding of the labeled chlorotoxin, wherein a high level of binding is indicative that the tissue is neoplastic. Preferably, the chlorotoxin is selected from the group consisting of native, synthetic and recombinant chlorotoxin. Preferably, the labeled chlorotoxin is radiolabelded and the level of radiolabeled chlorotoxin binding affinity indicative of neoplastic tissue is from about 5 nM to about 5 micromolar. The radiolabeled chlorotoxin may be, e.g., ^{131}I-chlorotoxin or ^{125}I-chlorotoxin. Alternatively, the chlorotoxin is labeled with a fluorescent moiety and the fluorescently labeled chlorotoxin binding is determined by a method selected from the group consisting of fluoresecent microscopy and fluorescent activated cell sorting. The radiolabeled chlorotoxin binding may be determined, for example, using positron emission tomography scanning.

The present invention is also directed to a fusion protein, said protein comprised of: a ligand that specifically recognizes an antigen in chloride channels of glial-derived tumor fused to a cytotoxic moiety. In one embodiment, the ligand is a chlorotoxin-like protein. In another embodiment, the ligand is an antibody. Representative cytotoxic moieties include gelonin, ricin, saponin, pseudonomas exotoxin, pokeweed antiviral protein, diphtheria toxin, and complement proteins.

The present invention is also directed to a pharmaceutical composition, comprising the fusion protein of the present invention and a pharmaceutically acceptable carrier. The present invention is also directed to a method of treating an individual having a glioma or meningioma, comprising the step of administering to said individual a pharmacologically effective dose of any of the compositions of the present invention.

It is specifically contemplated that pharmaceutical compositions may be prepared using the novel antibodies and fusion protein of the present invention. In such a case, the pharmaceutical composition comprises the novel antibodies and fusion proteins of the present invention and a pharmaceutically acceptable carrier. A person having ordinary skill in this art would readily be able to determine, without undue experimentation, the appropriate dosages and routes of administration of the novel antibodies and fusion proteins of the present invention.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Primary Cultures of Human Astrocytomas

(UAB Brain Tumor Research Laboratories, see Table 1 for details): Freshly resected brain tumor tissue was transported in ice-cold tissue culture medium and necrotic/hemorrhagic portions were removed aseptically. Discrete pieces of tumor tissue were minced finely, triturated, and plated in DMEM/F12 (Dulbecco's modified Eagle's medium mixed equally with Ham's Nutrient Mixture F12 supplemented with 10 mM HEPES, 2 mM L-glutamine) with 20% Fetal Bovine Serum (FBS, Atlanta Biologicals). Cells from minced fragments were replated onto uncoated 12 mm round coverslips for electrophysiology and for GFAP immmunocytochemistry. Acutely isolated tumor cells were prepared from fresh biopsy material, as described above with an additional trypsinization step in order to remove cellular debris, and were used for recordings 15–18 hours after plating.

EXAMPLE 2

Cell Lines

STTG1 cell line (American Type Culture Collection, Rockville, Md.) was grown in DMEM (Gibco) plus 10% FBS (Hyclone). Human Tumor Cell Lines: established cell lines, derived from human malignant gliomas (D54MG, U105MG, U251MG, and U373MG obtained from D. D. Bigner, Duke University) and extraglial human tumors (all from ATCC), were studied in long term (>100) passages (see TABLE I for details). Cells were maintained in DMEM/F12 supplemented with 7% heat-inactivated FBS (Atlanta Biologicals) at 37° C. in a 10% CO_2/90% air atmosphere. Cells attaining nearly confluent growth were harvested and replated onto uncoated 75 cm^2 flasks or uncoated 12 mm circular glass coverslips for electrophysiology and were used 36–72 hours after plating, unless otherwise noted. Viable cell counts were determined by trypan blue exclusion.

TABLE I

Primary cultures and established astrocytoma cell lines

Cell Line Designation	Cell Type	Passage	GFAP	Cl- Current
Primary cultures				
UAB4630	GBM	1	unk	8/8
UAB8553	GBM	1	+	6/6
UAB12983	low-grade astrocytoma	1	+	7/7
UAB4613	pilocytic astrocytoma	1	+	6/6
UAB4663	pilocytic astrocytoma	1	+	5/5
UAB4720	anaplastic ependymoma	1	+	5/5
UAB485923	medulloblastoma	0	unk	10/10
Cell Lines				
CH-235MG	GBM	>100	+	18/18
D-54MG	GBM	>100	+	11/11
SK-MG-1	GBM	>100	+	10/10
STGG1	anaplastic astrocytoma	>100	+	470/470
U-105MG	GBM	>100	+	10/10
U-251MG	GBM	>100	+	28/28
U-373MG	GBM	>100	+	10/10

Code:
GBM = glioblastoma multiforme;
+ = > 70% positive;
unk = unkown

EXAMPLE 3

Biopsy Tissue

Freshly resected human brain tumor tissue are collected during surgery in ice-cold tissue culture medium and necrotic/hemorrhagic portions are removed aseptically. Tissue is maintained for <15–20 min under 95/5% O_2/CO_2 until used for slicing. Ice-cold tissue are embedded in BactoAgar and cut into blocks of ~10×10 mm and glued to the bottom of a petri dish mounted to a Vibratome where 200 mm slices are cut. These are transferred to oxygenated saline and maintained at 37° C. until recording.

EXAMPLE 4

Xenografted Tumors in SCID Mice

C.B.-17 SCID mice are anesthetized by intraperitoneal administration of the following mixture: ketamine, 20 mg/ml plus xylazine, 0.3 mg/ml, in saline, at 0.07 ml/10 g of body weight. A midline scalp incision is made and a 0.5 mm burr hole is made at 1.5–2 mm to the right of the midline and at 0.5–1.0 mm posterior to the coronal suture. Tumor

cells (10^6 D54 MG- human glioma cells in 5 ml final injection volume/ mouse) are suspended in excipient (serum free DMEM/F12+5% methyl cellulose). Intracranial injection is performed stereotactically using a 250 ml Hamilton syringe with a 30-gauge needle mounted on a Stoelting stereotaxic apparatus. The needle is inserted vertically through the hole to a depth of 2.5 mm. 45–60 seconds after injection, the needle is slowly withdrawn and the incision closed with 9 mm Michel wound clips. Mice are then returned to sterile microisolator polycarbonate cages placed over a heating pad until recovery, and provided autoclaved lab chow and sterile water a d libitum. Slices are obtained from anesthetized mice after decapitation. The brain is quickly removed and placed in ice-cold (4° C.) calcium-free ringers containing (in mM): NaCl 116; KCl 4.5; $MgCl_2$ 0.8; $NaHCO_3$ 26.2; glucose 11.1; N-2-hydroxyethlypiperazine-N'-2-ethanesulfonic acid (Hepes) 5. The solution is constantly bubbled with 95% O_2/5% CO_2 mixture. The brain is hemisected and mounted onto a vibratome slice-holder using cyanoacrylate glue. Transverse tissue slices (50–200 mM) are cut in cold oxygenated saline solution and subsequently transferred to a beaker filled with Ca containing saline at room temperature.

EXAMPLE 5

Electrophysiology

Current and voltage recordings were obtained using standard whole-cell patch-clamp techniques with an Axopatch-1D amplifier (Axon Instruments). Patch-pipettes were made from thin-walled borosilicate glass (WPI, TW150F-40) o.d. 1.5 mm, i.d. 1.2 mm and were filled with a solution containing (in mM): KCl 145, $MgCl_2$ 1, $CaCl_2$ 0.2, EGTA 10, Hepes 10, pH adjusted to 7.4 using Tris, unless otherwise noted. Pipettes were not fire-polished and typically had resistances between 2–5MΩ. Cells were continuously superperfused with saline solution, allowing for rapid (<30 seconds) exchange of bath volume. The standard bath solution contained, in mM: NaCl 122.6, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1.0, Na_2HPO_4 2.0, NaH_2PO_4 0.4, $NaHCO_3$ 25.0, Na_2SO_4 1.2, Glucose 10.5 (bubbled with 5% CO_2/95% O_2). The composition of bath solutions used for replacement studies is summarized in Table II. Drugs used to block ionic conductances were prepared freshly as stock solutions for each experiment and added to bath solution. Osmolality was measured with a vapor pressure osmometer (Wescor, Logan, UT) and adjusted to 308–312 mOsms.

used as the value for the whole-cell membrane capacitance. Series resistances, monitored at regular intervals throughout each experiment, were usually 5–10MΩ, and series resistance compensation was typically set to ~80%. Entrance potential, read from the amplifier at the time of entering the whole-cell configuration, was used to determine each cell's resting potential. Voltage-clamp recordings were used to search for voltage-activated currents and stimulation profiles were altered to fully activate chloride channels (pulses from −105 to 195 mV). Where indicated, P/4 leak subtraction was obtained using hyperpolarizing voltage steps to obtain leak currents. Current reversal potential (voltage at which I=0) was determined from IV plots in which tail current amplitudes were plotted as a function of voltage. Effects of channel blockers were assessed by comparing current traces, entrance potential, and reversal potential prior to and following drug application. Snap photographs were taken of each recorded cell using a CCD camera and a video printer for cataloging of cell size, location, and morphology. Recordings were made at room temperature, typically 20–25° C.

EXAMPLE 6

Proliferation Assay

Proliferation was studied quantitatively by determining incorporation of ^{3}H-thymidine. In brief, cells were incubated for 24 hours in the continuous presence or absence of Ara-C (cytosine arabinoside, 10 μM), DIDS (200 μM), DNDS (200 μM), zinc (200 μM) or chlorotoxin (600 nM). Cells were incubated with 1 μCi/ml radiolabelled thymidine ([methyl-^{3}H]thymidine) for the final 4 hours (at 37° C.). Culture dishes were rinsed three times with ice-cold PBS and solublized with 0.3N NaOH for 30 minutes at 37° C. One aliquot (50 ml) was used for cell protein determination using the bicinchroninic assay (BCA; Pierce Rockford, Ill.). The remaining cell suspension was mixed with Ultima Gold, and radioactivity was determined with a scintillation counter. The results were expressed as cpm/μg protein.

EXAMPLE 7

Data Analysis

The theoretical equilibrium potentials were calculated according to the Nernst equation. The ion activities were adjusted from the ion concentrations used in solutions using activity coefficients obtained from Robinson and Stokes (34), which were 0.888, 0.886, and 0.888 for $[Na^+]$, $[K^+]$, and $[Cl^-]$, respectively. Calculated equilibrium potentials

TABLE II

Composition of external solutions (in mM)

External Solution	Na^+	K^+	HCO_3^-	Hepes	Ca^{2+}	Mg^{2+}	EGTA	Cl^-	Br^-	F^-	I^-	NO_3^-	Isethionate	glutamate	acetate	sucrose	glucose
HCO_3^-	122	5	25	—	1	1.2		132									10.5
Hepes	125	5		32.5	1	1.2		132									10.5
NaBr	125	5		32.5	1	1.2		132	125								10.5
NaF	125	5		32.5	1	1.2		132		125							10.5
NaI	125	5		32.5	1	1.2		132			125						10.5
$NaNO_3$	125	5		32.5	1	1.2		132				125					10.5
Isethionate	125	5		32.5	1	1.2		132					125				10.5
Glutamate	125	5		32.5	1	1.2		132						125			10.5
NaAcetate	125	5		32.5	1	1.2		132							125		10.5
0 NaCl	0	5		32.5	1	2.2	5	7.4								250	10.5

For whole-cell recordings, cell capacitance compensation and series resistance compensation were used to minimize voltage errors. The amplifier reading of capacitance was under the imposed ionic gradients in control solution were E_K=−83.4 mV, E_{Na}=+62.6 mV, and E_{Cl}=+2.8 mV. For all experiments, mean values and standard deviation (SD) were

computed from raw values entered into a spreadsheet (Excel, Microsoft). These data were exported to a scientific graphing and data analysis program (ORIGIN, MicroCal). Data were graphed as mean±S.E.M. Statistics were computed from raw data. For physiological effects of channel blockers, a paired, one-tailed t-test was used. For proliferative effects of channel blockers, results were analyzed using ANOVA test for multiple comparisons. DIDS (4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid), DNDS (4,4'-Dinitrostilbene-2,2'-disulfonic acid), Ara-C, and all other drugs were all purchased from Sigma. Chlorotoxin was purchased from Lanoxin (Accurate Chemical and Scientific Corp., Westbury, N.Y.).

EXAMPLE 8

Results

Whole-cell voltage clamp recordings were obtained from primary cultures of 7 freshly resected primary human brain tumors. In addition, a human anaplastic astrocytoma cell line, STTG1, was studied. The majority of STTG1 and primary-cultured cells were positive for glial fibrillary acidic protein (GFAP). Cells chosen for recordings were typically alone or isolated from other cell clusters and displayed bipolar, fibroblast-like morphology. Under normal recording conditions, time- and voltage-dependent outward currents were observed in all (N=490) recorded STTG1 astrocytoma cells and in all recorded primary cultured astrocytoma cells (N=60). Recordings from acutely isolated tumor cells were also obtained within 15–18 hours of plating (UAB485923, N=10). Currents were qualitatively similar in all preparations. The resting potential, determined as the entrance potential with KCl-containing pipette solution, was −14.1 mV (N=490, SD=14.6, SEM=0.66) and −20.15 mV (N=60, SD=17.54, SEM=2.28), in cell lines and primary cultures, respectively.

EXAMPLE 9

Chloride Currents in Human Astrocytoma Cells

Representative examples of whole-cell recordings from an STTG1 human astrocytoma cell and an astrocytoma cell from primary culture (UAB4630) in response to depolarizing voltage steps are displayed in FIG. 1. The cells were stepped from a holding potential of 0 mV to a series of test potentials between −105 mV and 195 mV in 25 mV increments. Potential >45 mV resulted in fast activating, non-inactivating outward currents. Cells showed large outward transients upon termination of voltage steps (FIGS. 1A and C). The IV relation plotting peak current amplitude as a function of voltage (FIGS. 1B and D) showed pronounced voltage dependence and outward rectification for both the transients (FIGS. 1 B, D "*") and steady-state currents (FIGS. 1B, 1D "x"). Mean conductance of 36 primary cultured cells was 5.67 nS (SD=4.62, SEM=0.77) and of 50 STTG1 cells was 5.29 nS (SD=3.63, SEM=0.51) (determined at 145 mV). To account for differences in cell size, values were normalized to membrane capacitance, yielding specific conductances of 195 pS/pF and 208 pS/pF, respectively. To determine the ion species that was carrying the outward current, the reversal potential of tail currents were analyzed. Therefore, cells were held at 0 mV, pulsed to 180 mV, and then stepped in −20 mV increments from +120 mV to −20 mV (FIG. 2A). Plotting tail currents as a function of voltage showed a reversal potential of 8 mV (FIG. 2B) in this example. Analysis of 12 primary cultured cells yielded a mean reversal potential of 0.1 mV (SD=11.3) and of −4.6mV (N=48, SD=14.1) in STTG1 cells. Under the imposed ionic gradients (E_{Cl^-}=+2.8, E_{K^+}=−83.4, E_{Na^+}=+62.6 mV), this is compatible with a reversal potential expected for either a Cl^--selective current or a nonselective cation current. Cells from all studied primary cultures and all STTG1 cells displayed such outwardly rectifying currents, and subsequent analysis did not distinguish between these two preparations.

EXAMPLE 10

Channel Selectivity for Cl^-

In order to determine the ion selectivity of the outward current, all but 7 mmol/L of the Cl^- in the bath solution was substituted with the sodium salts of a number of other monovalent anions (See Table II for composition of solutions), while keeping the pipette [Cl^-] constant (147.4 mM). To facilitate ion replacement studies, Hepes-buffered solutions were used; changing to Hepes-buffered solution as compared to HCO_3^--buffered solution by itself did not alter currents, suggesting that HCO_3^- does not permeate the channel under these conditions. Recordings obtained in Hepes- and HCO_3^--buffered external solutions were virtually indistinguishable, with no change in current amplitude or tail current reversal potential (data not shown).

FIG. 3 shows examples of whole-cell leak-subtracted current responses of human astrocytoma cells to test pulses stepped from a holding potential of 0 mV to 145 mV prior to and following substitution of bath chloride with the halide anions bromide (A), iodide (B), nitrate (C), and fluoride (D). Bromide, iodide, and nitrate increased outward currents, whereas fluoride substitution led to decreased currents. For each experiment, complete IV curves were plotted in (E). To compare I-V relations, currents were normalized to control currents with Cl^- as the external anion as the membrane was stepped from 0 mV to a series of potentials between −105 and +195 mV. Largest currents in Cl^--containing control solution were arbitrarily defined as 1. Currents in iodide and nitrate exceeded Cl^- currents by >2-fold. Similarly, FIG. 4 shows the whole-cell leak-subtracted current responses with the same experimental protocol as in FIG. 3 prior to and after substitution with (A) acetate, (B) glutamate, (C) isethionate, and (D) sucrose. Acetate and isethionate led to decreased outward currents, while glutamate and sucrose virtually eliminated outward currents. The current voltage relations for the non-halide substitutions normalized to normal NaCl-rich bath solution are shown in part (E). The selectivity for the different anions was calculated from the shift of the reversal potential under the imposed ionic gradients according to the Goldman-Hodgkin-Katz equation:

$$\Delta E_{rev}=E_{rev.anion}-E_{rev.\ Cl}=(RT/zF)\ln(P_{anion}[\text{anion}]_o/P_{Cl}[\text{Cl}]_o,$$

where R, T, and F have their usual meanings. In this calculation, it was assumed that the currents measured under the conditions of the experiment were carried solely through Cl^- channels. In total, the permeability of seven different anions was tested. Table III summarizes the changes in the values of E_{rev} for equimolar replacement of chloride by test anions and the calculated permeability ratios (P_{anion}/P_{Cl}) These data suggest the following relative permeability sequence:

$I^->NO_3^->Br^->Cl^->$acetate>isethionate>$F^->$glutamate.

TABLE III

		Anion selectivity.		
Na⁺-Anion	MW	ΔE_{rev}(mV)	P_{anion}/P_{Cl}	N
Chloride	58.45	—	—	48
Acetate	82.04	4.0 ± 0	0.90 ± 0.02	3
Bromide	102.9	−15.5 ± 6.3	1.95 ± 0.47	3
Fluoride	42.0	24.6 ± 3.0	0.41 ± 0.05	3
Glutamate	169.1	29.8 ± 2.3	0.33 ± 0.18	2
Iodide	149.9	−20.8 ± 7.5	2.44 ± 0.65	5
Isethionate	148.1	18.5 ± 0.7	0.51 ± 0.01	3
Nitrate	85.01	−15.8 ± 8.8	2.02 ± 0.58	4
(Sucrose)	342.3	32.2 ± 17.8	—	3

Table 3: Anion selectivity—The reversal potential (E_{rev}) was determined in each test solution by plotting peak tail current amplitude against the applied voltage step after cells were stepped to 180 mV and then brought from +120 mV to −120 mV in −20 mV increments. Results are expressed as the mean change in E_{rev} from control solution containing NaCl. The permeability ratio P_{anion}/P_{Cl} was determined using the Goldman-Hodgkin-Katz equation.

EXAMPLE 11

Effect of Cl^- Channel Blockers

The outward current pharmacologically were further characterized by examining the effect of several established Cl^- channel blockers, including chlorotoxin, DIDS, and DNDS. FIG. **5** shows representative whole-cell leak subtracted traces and current-voltage relations before and after bath addition of chlorotoxin, DIDS (4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid) and DNDS (4,4'-Dinitrostilbene-2,2'-disulfonic acid). Bath application of 590 nM chlorotoxin reduced both the steady state and transient amplitude evoked by voltage steps from −105 to +195mV by 81.9%±0.88 (n=4) of the control value (FIGS. 5A–C). This effect was partially reversible. Chlorotoxin was also effective at higher concentrations in blocking Cl^- currents when applied to the cytoplasmic face (60.44% at $[\text{chlorotoxin}]_i$=2.5 μM, N=7, SD=17.8, data not shown). Chlorotoxin is a protein having 36 amino acids that is derived from scorpion venom toxin that was originally described as a blocker of small conductance Cl^- channels in epithelial cells (8). In order to ensure that the effects of chlorotoxin did not result from any contaminants in the venom toxin, the peptide was synthesized and comparable inhibition of currents with the synthetic toxin were observed (data not shown). As above, currents are shown before and after application of 100 μM DIDS (FIGS. 5D, 5E) and 100 μM DNDS (FIGS. 5G, 5H). Current-voltage relations from those examples are shown in parts (F) and (5I). The size of the outward current was reduced by DIDS at all potentials (33.5%±12.9(n=5)). Similar to DIDS, DNDS caused a decrease in current amplitude at all potentials by 38.2%±13.3 (n=4). DIDS and DNDS were more effective in blocking currents when applied to the cytosolic face, albeit at higher concentrations (200 μM, 50%±10.9 (N=3) and 62% (N=1), respectively, data not shown). The action of both drugs was partly reversible with short exposure times, though the recovery was never complete.

The effects of the heavy metals zinc and cadmium on outward currents were also examined. These drugs have been shown to block Cl^- currents in T lymphocytes (35) and Schwann cells (33).

Bath application of 100 μM zinc led to a 47%±25.9 (n=3) decrease in peak currents (FIGS. **6**, A–C), and 25 μM cadmium led to a 42%±18.5 (n=5) decrease (FIGS. **6**, D–E). Since Cd^{2+} is also a blocker of voltage-dependent Ca^{2+} channels, it is possible that reduced currents may have resulted indirectly from reducing Ca^{2+} influx. To help elucidate whether this may have been the case, a bath solution was applied in which all Ca^{2+} had been removed, with the addition of 5 mM EGTA. In a zero calcium environment, currents were decreased to 42.6%±16.8 (n=5) of that in control solution, containing 1 mM Ca^{2+} (FIGS. **6**, F–H), suggesting that, indeed, Cl^- currents are at least partially dependent on $[Ca^{2+}]_o$. A summary of the pharmacological effects on current amplitude is shown in FIG. **7**, with the values expressed as percent of control current in standard external solution. Based on the ion replacement studies and pharmacology, it was concluded that the outwardly rectifying currents were mediated by anions. Under physiological conditions, the current would be carried by Cl^-, thus it can be referred to as a Cl^- current.

EXAMPLE 12

Cl^- Channels and Astrocytoma Proliferation

Given that these Cl^- currents were consistently present in all astrocytoma cells tested from both primary cultures of surgical specimens and from established human astrocytoma cell lines, whether Cl^- currents influence astrocytoma proliferation was examined. Although the effects of Cl^- channel blockade on cell proliferation have been reported in Schwann cells (40) and in B lymphocytes (7), the importance of Cl^- ion channels in glia cells has never been shown. Cells were cultured in the continuous presence of the anti-mitotic agent Ara-C (10 μM), DIDS (200 μM), DNDS (200 μM), Zinc (200 μM), or chlorotoxin (600 nM) and compared the rate of proliferation to untreated (control) sister cultures. Cells were treated at 2 days in culture (DIC) and proliferation was assayed 24 hours later, at 3 DIC, a period of high proliferation of untreated control cultures. As expected, incubation in the anti-mitotic agent Ara-C led to a 70% decrease in proliferation (SD=1.3309, N=17). The putative chloride channel blockers DIDS, DNDS, and zinc decreased proliferation by 16.4%(SD=20.0, N=16), 38.2% (SD=13.1, N=8), and 72.6% (SD=12.4, N=7), respectively. By contrast, incubation in either the native or synthetic venom toxin chlorotoxin led to an increase in proliferation compared to control (mean=37.8%, SD=5.7, N=8 and mean=28.4%, SD=16.34, N=9) respectively).

The present invention identified a voltage-dependent, outwardly-rectifying Cl^- current in human astrocytoma cells. This current was present in all cells studied in both primary cultures of human astrocytomas and in an established human astrocytoma cell line. Cells showed large outward transients upon termination of voltage steps and reversed close to the calculated equilibrium potential for chloride. Upon replacement with various anions, the current reversal potential shifted in accordance with an anion-selective channel towards the new E_{Cl}. Currents were sensitive to application of chloride channel blockers chlorotoxin, DIDS, DNDS, cadmium, and zinc. Under physiological conditions, the current would be carried by Cl^-, so that currents were considered chloride currents. The presence of the current was surprising in light of the fact that non-neoplastic glial cells are typically characterized by high levels of expression of voltage-gated K^+ channels; no appreciable contribution from K^+ currents to whole-cell outward currents was observed in all cells tested.

Outwardly rectifying chloride currents have been described in many epithelial tissues including respiratory cells (24), submandibular gland (19), lacrimal gland (9), pancreatic duct cells (18), epididymis (31), and sweat gland (23), and in non-epithelial cells such as lymphocytes (11), squid axon (18), and rat skeletal muscle (3). The physiological function of these outwardly rectifying channel in cell types other than secretory epithelia remains unclear. In the latter, they are believed to participate in transepithelial solute transport and volume regulation (10).

The current observed in astrocytoma cells, although similar to epithelial cells in its sensitivity to Cl^- channel blockers, shows several differences: First of all, in some preparations, such as fetal pancreas (13), fetal epididymis (31), and pancreatic ductal cells (2), chloride currents show little or no voltage dependence. Secondly, another class of chloride channels shows a peculiar voltage-dependence with activation near 0 mV and inactivation with potentials more than 20 mV in either direction (3,27,36). Astrocytoma Cl^- channels are strongly voltage-dependent at all potentials >50 mV. In this regard, they are most similar to Cl^- channels found in human macrophages(16), necturus enterocytes(12), squid axon(18) and sheep parotid gland (19). Thirdly, in some cell types, such as colon muscle (1), submandibular gland (19), rat muscle (3), and A6 epithelia cells (27), chloride channels do not show spontaneous activity in whole cell recordings and channel activation occurs only in excised patches. In contrast, astrocytoma Cl^- currents could be easily recorded in every recording in the whole-cell configuration.

The permeability sequence of the chloride channel in astrocytoma cells does not correlate with the hydrated ion radii ($NO_3^- > Cl^- > I^- > Br^-$) or the mobility of ions in aqueous solution ($Br^- > I^- > Cl^- > NO_3^-$). The sequence most closely resembles the lyotrophic series ($I^- > NO_3^- > Br^- > Cl^- > F^-$), which reflects the ability to denature macromolecules or to bind or absorb to proteins or lipid-water interfaces (6). The anion selectivity sequence here differs in only minor detail from those reported for outwardly rectifying channels in other tissues: submandibular duct gland ($SCN^- > NO_3^- > I^- > Cl^- > Br^-$>acetate)(19), canine airway epithelia ($SCN^- > NO_3^- > I^-$ $Br^- > NO_3^- > Cl^-$) (25), rat lacrimal gland ($I^- > NO_3^- > Br^- > Cl^- > F^-$>isethionate>glutamate) (9) and necturus enterocytes ($SCN^- > I^- > Br^- > Cl^- > F^-$>gluconate) (12). Typically, replacement of Cl^- by large organic anions results in the virtual abolishment of Cl^- currents. Similarly, in the recordings herein, currents were almost eliminated after glutamate or sucrose replacement.

Brismar and Collins (1989) tested various human astrocytoma cell lines and found a high density of inwardly rectifying potassium channels active at or near resting potential. The current component active at potentials more negative than 0 mV was blocked by Cs^+ and was dependent on $[K^+]_o$, such that replacement with high K^+ solutions led to an increase in the inward currents. Upon closer examination, the current component active at potentials more positive than 0 mV was insensitive to ion replacements of Na^+ or K^+ and was also insensitive to Cs^+ blockade. This is the range of voltage steps that produces an IV relation most similar to the one observed here.

These authors did not further investigate the current contributions >0 mV. No appreciable contribution of K_{ir} currents was seen herein. It is possible that the cells' proliferative state or differences in culture conditions may alter the presence of K_{ir}. However, unlike Brismar and Collins, the present invention also examined cells prepared from primary cultures of surgical specimens from astrocytomas and did not see any appreciable K_{ir} currents.

Recently, a chloride current has been described in an astrocytoma cell line (U373MG) that is only activated by hyposmotic conditions but not present under normosmic conditions. These authors report that outward currents are sensitive to one of the chloride channel blockers used herein, namely DIDS, in addition to some additional putative channel blockers. Though the IV relations appear similar to those described in the same voltage range, currents do not show the large outward transients upon termination of the voltage steps characteristic. Most importantly, the currents in these cells were not active under normosmic conditions, and the cells must have been exposed to hyposmotic bath solutions before the chloride currents could be evoked. Again, these authors did not investigate cells from surgical specimens in their studies. In addition, the presence of an anion current in cultured rat cortical astrocytes has been recorded which is active only in 1–2 out of 100 excised patches in normosmic conditions and with increased frequency in hyposmic conditions (20). Whole cell Cl^- currents were previously recorded in cultured rat astrocytes; however, these currents differ markedly in their voltage dependency and relative permeability to anions from that described here for several reasons (14). The present invention discloses the presence of outwardly-rectifying, voltage-dependent chloride currents in biopsies prepared from surgical specimens from 6 different human astrocytomas and from 7 different established human astrocytoma cell lines (39) under normosmic conditions.

Furthermore, the present invention recorded Cl^- currents in the cell line U373MG in addition to other established astrocytoma cell lines (CH-235MG, D-54MG, SK-MG-1, U-105MG, U251MG. Moreover, the currents were observed in all cells under normal conditions, with osmolality of each solution measured and matched to the osmolality of the growth medium.

The precise role of this chloride conductance in astrocytoma cells is unclear. Ion channels have been shown to be part of the proliferative response in a number of cell types and in cultures of normal glial cells, the activity of K^+ channels is required for cell proliferation, since K^+ channel blockade leads to decreased proliferation. Potassium channels have been implicated in the proliferative response in a number of other cell types, including human melanoma cell lines (28), cultured brown fat cells (30), and Schwann cells, the principle glial cells in the peripheral nervous system (5). The present invention demonstrates that the link between channel activity and proliferation is more widespread. Modulation of channels may result from both long-term changes in gene expression and short-term modulation of pre-existing channel proteins.

A link between chloride channels and the proliferative response has only recently been suggested. In cultured B cells, the stilbene disulphonates and putative chloride channel blockers SITS and DIDS were found to be effective mitogens and directly stimulated proliferation (7). Moreover, the mitogenic responses to DIDS were routinely larger than those obtained with the B cell mitogen LPS. These experiments imply that there is a signal transduction pathway leading to cell proliferation that directly involves anion movement across the cell membrane. In Schwann cells, SITS and DIDS application leads to a 2- to 5-fold enhancement of proliferation in both unstimulated and mitogen stimulated proliferation (40). The present invention observed a decrease in proliferation by DIDS, DNDS, and zinc and a 37% enhancement of astrocytoma proliferation following application of chlorotoxin. One possible explanation is that the stilbene derivatives are affecting ion transport mechanisms, whereas chlorotoxin is a more specific ion

channel inhibitor. Thus, the present invention shows that Cl^- channels participates in the proliferative response in these cells.

EXAMPLE 13

Electrophysiology

For the following studies, the electrophysiology format was as follows: standard current and voltage recordings were obtained using the whole-cell patch-clamp technique with an Axopatch-1D amplifier (Axon Instruments). Cells were continuously superperfused with bicarbonate-buffered saline at room temperature containing, in mM: NaCl 122.6, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1.0, Na_2HPO_4 2.0, NaH_2PO_4 0.4, $NaHCO_3$ 25.0, Na_2SO_4 1.2, Glucose 10.5 (bubbled with 5% CO_2). Electrodes (WPI, TW150F-40) o.d. 1.5 mm, i.d. 1.2 mm were filled with (in mM): KCl 145, $MgCl_2$ 1, $CaCl_2$ 0.2, EGTA 10, Hepes 10, pH adjusted to 7.4 using Tris, unless otherwise noted. Entrance potential, read from the amplifier at the time of entering the whole-cell configuration, was used to determine each cell's resting potential. Voltage-clamp recordings were used to search for voltage-activated currents and stimulation profiles were altered to fully activate chloride channels (pulses from −120 to 120 mV). Current reversal potential (voltage at which I=0) was determined from IV plots in which tail current amplitudes were plotted as a function of voltage. Effects of channel blockers were assessed by comparing current traces, entrance potential, and reversal potential prior to and following drug application. Recordings were made at room temperature.

EXAMPLE 14

Ion Channel Expression in Human Astrocytoma Cells

Whole-cell voltage clamp experiments were performed on primary cultures and on established cell lines—both derived from human astrocytomas (see TABLE IV). All of the primary cultures and all of the cell lines studied (with the exception of one primary culture not tested) were >80% GFAP-positive. FIG. **9** shows typical whole-cell recordings from an anaplastic astrocytoma cell (STTG1). Depolarizing voltage steps activated time- and voltage-dependent outward currents in all (N=577) recorded astrocytoma-derived cells. The resting potential, determined as the entrance potential with KCl-containing pipette solution, was −14 mV(SD=15, SEM=0.62, N=577). Cells were stepped to test potentials between −120 mV and 120 mV in 20 mV increments from a holding potential of 0 mV. Cells showed large tail currents upon termination of voltage steps (FIG. **9**A). Potential >0 mV results in fast activating, non-inactivating outward currents. The IV relation plotting peak current amplitude as a function of voltage (FIG. **9**B) showed pronounced outward rectification. In order to determine the ion species that was carrying the outward current, the reversal potential of tail currents was analyzed. Therefore, cells were held at 0 mV, pulsed to 200 mV, and then pulsed in −20 mV increments from +120 mV to −120 mV (FIG. **9**C). Plotting tail currents as a function of voltage showed a reversal potential of 0 mV. Under the imposed ionic gradients ($E_{Cl'}$=2.8, EK^+=−83.4, ENa^+=67.3), this is compatible with that expected for either a Cl^--selective current or a nonselective cation current (FIG. **9**D).

TABLE IV

Primary cultures and established astrocytoma cell lines

Cell Line Designation	Cell Type	Passage	GFAP	Cl^-- Current
Astrocytomas				
UAB4630	GBM	1	unk	8/8
UAB8553	GBM	1	+	6/6
UAB12983	LGA	1	+	7/7
UAB4613	PA	1	+	6/6
UAB4663	PA	1	+	5/5
UAB4720	AE	1	+	5/5
CH-235MG	GBM	>100	+	18/18
D-54MG	GBM	>100	+	11/11
SK-MG-1	GBM	>100	+	10/10
STGG1	AA	>100	+	470/470
U-105MG	GBM	>100	+	10/10
U-251MG	GBM	>100	+	28/28
U-373MG	GBM	>100	+	10/10
Non-glial tumors				
SK-MEL-3	mela.	5	−	0/10
MCF-10A	N.B.	41	−	0/5
MCF-7	B.CA	155	−	0/12
TE671	R.	17	−	0/12
IMR-32	R.	57	−	0/6
SK-N-SH	R.	53	−	0/5

Code:
GBM = glioblastoma multiforme;
LGA: low grade astrocytoma;
PA" pilocytic astrocytoma;
AE: anaplastic ependymoma;
AA: anaplastic astrocytoma;
mela.: melanoma;
N.B.: normal breast;
B.CA: breast cancer;
R. rhabdomyosarcoma;
+ = > 80% positive;
unk = unkown

EXAMPLE 15

Cancer Relevance

Glioma cells express a unique transmembrane Cl^- ion channel that binds a venom toxin (chlorotoxin) with very high affinity. The high affinity for chlorotoxin allow the development of glioma-specific agents including marker compounds for rapid diagnosis and immunotoxins for therapeutic treatment. Since this protein is only shared among malignant glioma cells and meningioma cells it could be targeted by reagents that bind to the toxin binding site, or, following isolation of the protein, antibodies could be used to selectively eliminate cells expressing this protein. This approach has a high likelihood to yield new strategies for more specific and more effective therapeutic modalities for this uniformly fatal disease.

EXAMPLE 16

Expression of GCC in Acute Patient Biopsies

Biopsies from 24 patients diagnosed with gliomas were thoroughly investigated histopathologically. Electrophysiological recordings and immunohistochemical methods were used to detect GCC. Expression was observed in all patients and spanning in age from 0.5 to 77 years and independent of pathological grade of the tumor. An example of a representative recording is shown in FIG. **10**. Evidence for the expression of GCC was obtained in 4/4 biopsies of patients diagnosed with meningioma. A list of patient cases studied in which GCC was identified is presented in TABLE V.

TABLE V

Case #	age	sex	tissue pathology	location	WHO grade	Slice/ Culture	# cells	Passage
1	5	F	pilocytic astrocytoma	hypothalamus	I	S/C	9/25	0
2	7	F	pilocytic astrocytoma	cerebellum	I	S/C	10/12	0
3	11	M	pilocytic astrocytoma	posterior fossa	I	C	10	0
4	3	M	pilocytic astrocytoma	cerebellum	I	C	3	0
5	14	F	pilocytic astrocytoma	thalamus	I	C	6	1
6	8	F	pilocytic astrocytoma	temporal lobe	I	C	5	1
7	4	M	pilocytic astrocytoma	temporal lobe	I	S/C	7/10	0
8	1	M	pilocytic astrocytoma	posterior fossa	I	S	6/—	—
9	0.5	M	pilocytic astrocytoma	posterior fossa	I	S/C	7/6	0
10	0.5	F	papilloma	ventricular	I/II	C	6	0
11	13	F	subependymalgiant cell astrocytoma	frontal lobe	I/II	S/C	12/8	0
12	56	F	low grade astrocytoma	parietal lobe	II	C	5	1
13	10	M	anaplastic ependymoma	occipital lobe	III	S/C	2/5	0
14	48	M	anaplastic oligo-dendroglioma	unknown	III	C	9	1
15	1	M	anaplastic ependymoma	parietal lobe	III	C	5	1
16	14	F	malignant (anaplastic) astrocytoma	periventricular, occipital	III/IV	S/C	12/4	0
17	69	M	GBM	temporal lobe	IV	C	5	1
18	4.5	F	GBM	cerebellopontine	IV	C	4	1
19	1.5	M	GBM	suprasellar, intraventricular	IV	S/C	8/10	0
20	77	F	GBM	frontal lobe	IV	C	6	0
21	66	F	GBM	temporal lobe	IV	C	11	0
22	0.5	M	medulloblastoma	posterior fossa	IV	C	6	0
23	3	M	medulloblastoma	posterior fossa	IV	C	6	0
24	44	M	desmoplastic medullo-blastoma	cerebellum	IV	C	5	1

GBM = glioblastoma multiforme;
"S" = slice only, "C" = culture only, "S/C" = both slice and culture preparations.

EXAMPLE 17

Experimental Tumors in SCID Mice

Glioma tumors experimentally induced in SCID mice were also studied by intracranial injection of D54MG glioma cells. This procedure resulted in rapidly growing, invasive brain tumors (Gladson et al. 1995) from which slice preparations were made. Close to 100% of cells in tumor xenografts showed prominent expression of GCC as illustrated by staining of tumor tissue with antibodies that recognize Ctx binding sites (FIG. **12**) or electrophysiology (FIG. **11**). An example of a representative recording from D54MG cells is shown in FIG. **11**. These scid mice were also used to study the biodistribution of Ctx binding sites (therefore GCC channels) using ^{125}I-Ctx. Therefore, ^{125}I-Ctx was injected into the cerebrum of a mouse in which a glioma had been induced in the right brain 14 days earlier. Brain and body tissue as well as blood was harvested and ^{125}I-Ctx levels were determined using a liquid scintillation counter. The resulting counts show the selective accumulation of ^{125}I-Ctx in the tumor (FIG. **13**).

Upon replacement of either intracellular or extracellular potassium ions with Cs^+, current amplitude and reversal potential were unchanged. Currents persisted, with altered amplitude, if extracellular chloride was replaced by Br^-, Fl^-, or I^-. Reversal potential shifts indicated that, of these halide ions, Br^- and I^- exhibited greater permeability than either Cl^- or F^-.

The current was further characterized pharmacologically by examining the effect of several established Cl^- channel blockers. FIG. **14** shows an 80% decrease in outward current by bath application of 590 nM chlorotoxin to an STTG1 cell. Similar effects were observed with bath or pipette applications of DIDS (100 mM) and DNDS (100 mM) (data not shown). Similar to the work above and based on the pharmacology and ion replacement studies, it was concluded that the outwardly rectifying currents were mediated by an anion. Since under physiological conditions, the current was carried by Cl^-, it was referred to as a Cl^- current.

Over 570 cells from primary culture of 6 intracranial tumor resections and 7 cell lines (N>12 cells each) were screened and this Cl^- current was identified in all cells studied. FIG. **3** shows representative examples of voltage-dependent outwardly rectifying anion currents from selected primary cultures and more established cell lines. Cells from primary cultures displayed outward currents that were similar in size, voltage activation, reversal potential, and sensitivity to chlorotoxin as cell lines. Currents were qualitatively similar in all of the 7 cell lines evaluated (U251MG, CH235MG, U373MG, U105MG, D54MG, SK-MG-1, (all glioblastoma multiforme) and STGG1 (anaplastic astrocytoma)). By contrast, such currents were never observed in cell lines derived from other human cancers, such as neuroblastoma, melanoma, breast carcinoma, or rhabdomyosarcoma (See FIG. **15** for representative current traces), nor in rat C6 glioma cells or in primary astrocyte cultures of rat spinal cord or hippocampus (results not shown).

The present invention is the first report of an outwardly-rectifying Cl^- current in human malignant glioma cells. Currents were characteristic of both primary cultures of freshly resected brain tumors and established astrocytoma cell lines. These currents were not present in several extra-glial human tumors such as melanoma, breast, rhabdomyosarcoma and neuroblastoma. Chloride currents were characteristic of cells from other preparations, including lymphocytes, submandibular gland, rat myotubes, and sweat gland. However, while the currents were similar in their sensitivity to chloride channel blockers, the Cl^- current in astrocytoma cells exhibits a higher threshold for current activation, had large positive tail currents not previously

reported, and could be easily recorded in whole-cell patches. Most interestingly, the present invention demonstrates that this Cl^- current is in all tumor cells studied of glial origin but not in normal non-malignant glial cells or in non-glial tumors.

The present invention demonstrates a chloride conductance unique to human astrocytoma and glioblastoma cells which is not present in human tumor cells of extraglial origin. This channel can be blocked physiologically by chlorotoxin, a scorpion venom known to block epithelial chloride channels. The presence of this chloride channel activity presents a diagnostic strategy to differentiate between glial and non-glial tumors.

EXAMPLE 18

Identification and Treatment of Gliomas

Using the teaching of the studies described supra, a person having ordinary skill in this art would readily be able to identify and treat glial-derived neoplastic conditions, i.e., gliomas, astrocytomas, and glioblastomas. For example, chlorotoxin is a 36 amino acid protein naturally derived from *leiurus quinquestriatus* scorpion venom. Using techniques well known in the art, one may prepare recombinant proteins specifically engineered to mimic the binding and action of the native toxin. For example, recombinant chlorotoxin may be synthesized in *E. coli* and by virtue of its high affinity binding to chloride ion channels on the surface of human glial-derived tumors, such recombinant chlorotoxin with an appropriate label are used to identify and isolate glial-derived tumors. Because of the high affinity of the chlorotoxin/channel interaction, a fusion protein such as a primary antibody can be used to stain cells using standard immunohistochemical methods. A GST protein which lacks an insert was also purified for use as a control. An antibody against the GST portion alone can be used as a secondary antibody. In addition, the physiological activity of the fusion protein can be examined by using the GST with no insert as an internal control.

The biological activity of the synthetic chlorotoxin is as effective for chloride ion channel blockade as the native venom toxin. Recombinant techniques are used to synthesize chlorotoxin in *E. coli* using a modified PGEX vector system and the toxin may be linked to various fusion proteins using common restriction sites: GST-chlorotoxin, GST-Ala_{10} linker-chlorotoxin, and GST-Ala_{20} linker-chlorotoxin. These contain no linker, 10 alanine amino acid linker and 20 alanine amino acid linker, respectively. Specifically, three pairs of overlapping oligonucleotides of chlorotoxin sequence deduced from the peptide sequence were synthesized with a HindIII cohesive sequence at the 5' end of the sense oligonucleotide and an EcoRI cohesive sequence following the stop codon at the 3' end of the sense sequence. Each oligonucleotide was phosphorylated by T4 polynucleotide kinase using ATP as a substrate. Nucleotides were annealed by heating and slow cooling. Annealed oligonucleotides were cloned into HindIII/EcoRI site of pGBHE vector (pGCT-1) through ligation followed by transformation into *E. coli* competent cells. Similarly, a 20 amino acid linker was cloned into the BamH1/HindIII site. This amino acid linker has a BglII site in the middle that makes it possible to cut the BamHI and BglII in order to create a 10 amino acid linker sequence. The orientation and preservation of the oligonucleotide has been verified within the fusion protein by sequencing methods and that the induction of fusion protein produces the expected size was verified by comparing their molicular weights on a 12% SDS-PAGE gel.

After synthesis of recombinant chlorotoxin, it may be linked to various cytotoxic fusion proteins including glutathione-S-transferase (GST), gelonin, ricin, diptheria toxin, complement proteins and radioligands and other such proteins as are well known in the immunotoxin art. Thus, recombinantly prepared synthetic chlorotoxin linked to a cytotoxic moiety would be useful to specifically target and deliver a toxic substance to glial-derived tumors as a novel therapy. For example, GST-chlorotoxin fusion protein may be prepared as follows. Three fusion proteins, GST alone, GST-chlorotoxin, and GST-Ala_{20} linker-chlorotoxin were affinity purified using a glutothione conjugated agarose bead column and the resulting proteins were verified on a 12% SDS-PAGE gel. More specifically, *E. coli* were transformed with the vector and chlorotoxin insert and were induced to produce the fusion proteins. Resulting proteins were mixed with glutothione agarose beads and left for 15' to optimize absorption. Columns were washed with buffer and the fusion proteins were eluted by competition with free glutathione and collected in small vials. These proteins were then run on a 12% SDS-PAGE gel.

EXAMPLE 19

One Step Conjugation of DTAF (Dichlorotriazinylaminofluorescein) to GST-Ctx Fusion Protein

Gluthathione column purified fusion protein Ctx-GST is diluted in 0.2M sodium carbonate (pH 9.0), at 1–2 mg/ml. DTAF (Calbiochem) is diluted in 1.0 M sodium carbonate (pH 9.0) at 2.5 mg/ml. DTAF is mixed gently with the diluted Ctx-GST, by adding 25 mg DTAF per milligram of Ctx-GST. Mixing continues at room temperature for 10 minutes, after which NH_4Cl is added at a final concentration of 50 mM and glycerol up to 5% final volume (optional, xylene cylanol 0.1% is added to serve as indicator dye for the unbound material). The solution is placed at 4° C. for 2–4 hours with gentle agitation. After mixing, the unbound dye is separated by gel size filtration (G-Sephadex column, with exclusion limit between 30,000–50,000 prepared according to the manufacturer's instructions (Pharmacia)). The conjugated Ctx-GST-DTAF elutes first, and its color is easily distinguished under room light. Protein content is then determined, and the fluorescent conjugate is stored in a light proof-container at 4° C., until ready to use for direct immunofluorescence labeling of cells or slices as described below.

EXAMPLE 20

Chlorotoxin Binding Identified by Immunohistochemistry

GST-chlorotoxin (Ctx-GST) or Ctx-GST-DTAF are used to identify toxin binding sites. Ctx-GST is biologically active, binds to and blocks Cl^- channels with similar affinity as the venom toxin. Ctx-GST are recognized immunohistochemically by an antibody to GST (Chemicon) conjugated to either rhodamine or FITC, and binding are assayed under a fluorescence microscope. Alternatively, a single step fluorescence staining procedure are used utilizing Ctx-GST-DAFT (above), a fluorescent form of the Ctx-GST. The DTAF label can be visualized by direct immunofluoresence using standard FITC filters. The Ctx-GST-DTAF staining has the advantage that cross-reactivity with native GST does not pose a problem.

Antibodies to the chloride ion channels in glial derived tumors may be prepared as follows. Polyclonal antisera are generated by injecting fusion proteins created between the glutathione-S-transferase and the chlorotoxin insert into mice or rabbits. Mice are immunized with 0.5 ml of a 1:1 emulsion of 1 mg/ml purified fusion protein in Freund's complete adjuvant and subsequently with two additional injections after 14 and 28 days in Freund's incomplete

adjuvant. The mouse and rabbit antibodies are purified from the antisera using the GST fusion protein immobilized on nitrocellulose filters. The antibodies are then examined for binding specificity in various tissues.

EXAMPLE 21

Glioma Cells Bind ^{125}[I]-Chlorotoxin with High Affinity and Selectivity

To utilize Ctx-like molecules to selectively target glioma cells, it is essential to establish the selective high affinity binding of Ctx to glioma cells. Therefore, binding affinity was determined using (^{125}[I]-Ctx) radiolabeled with ^{125}[I]-sodium iodide by the chloramine-T method. Saturated binding was achieved in D54MG glioblastoma cells at concentrations >15 nM (FIG. **17**, inset). Scatchard analysis of these data indicates two binding sites with estimated binding affinity values (Kd) of 4.2 nM and 660 nM. The latter value is in good agreement with the electrophysiologically determined IC_{50} of ~950 nM. D54MG cells contain approximately 1,300 high affinity Ctx binding sites and 13,300 low affinity binding sites per cell. By contrast, no specific Ctx binding was observed in normal human glial cells nor in mixed brain cell cultures (not shown), suggesting that in brain, Ctx binding is glioma specific. This observations suggest that Ctx with radioactive moieties can be used to treat gliomas. The molecule would selectively bind to gliomas and expose cells to high levels of radiation. ^{125}I-Ctx or 131I-Ctx are candidates for this purpose.

EXAMPLE 22

Immunohistochemical Detection of Chlorotoxin

While electrophysiology is the perfect tool to detect channel activity, it cannot show the presence of inactive or quiescent channels. Other means to detect GCC channels are thus desirable and are particularly important for use of GCC as a diagnostic marker. Chlorotoxin binding can be detected immunohistochemically using several approaches. First, cells can be labeled with Ctx-GST, the fusion protein that also inhibits GCC currents (see above, FIG. **16**). This fusion protein can be detected by a FITC-conjugated antibody to GST, although numerous other detection procedures would be possible. As shown in FIG. **18**, this approach selectively labels astrocytoma and glioblastoma derived cell lines such as STTG1 or D54MG, but fails to label normal human glial cells. These studies, obtained in vitro, demonstrate the ability to use this approach for the detection of Ctx binding sites and can be used as a diagnostic marker for gliomas in human biopsies. Secondly, chlorotoxin can be labeled using DTAF as Ctx-DTAF. This procedures resulted in a directly fluorescent Ctx-molecule that selectively labels gliomas. Similarly, chlorotoxin can be directly conjugated with biotin as Ctx-biotin. This allows binding to be identified using a reaction with avidin and subsequent recognition by antibodies or the reaction product. This approach was likewise successful in selectively labeling glioma and meningioma cells.

EXAMPLE 23

Immunotoxins Targeted to the Ctx Binding Site can Specifically Kill Glioma Cells In Vitro

Since Ctx-GST selectively labels glioma cells, one may target and eliminate tumor cells by conjugating Ctx-GST to a known immunotoxin, e.g., saporin (Benatti et al. 1989; Battelli et al. 1990; Fordham-Skelton et al. 1990; Tecce et al. 1991; Fordham-Skelton et al. 1991). Such chimeric proteins can be made by fusing Ctx (the targeting moiety) with saporin (the toxin moiety).

Using this approach, glioma cells were first treated with Ctx-GST, followed by a mouse anti-GST monoclonal antibody and lastly a goat anti-mouse antibody conjugated to saporin. This last step confers immunotoxicity on the "primary" (in this case, Ctx-GST) antibody and resulted in significant and specific killing of glioma cells (FIG. **19**). Normal nontumor human astrocytes were not influenced by treatment with the saporin conjugate and either antibody alone failed to reduce cell numbers or to reduce protein or DNA synthesis, as assayed by ^{3}H-leucine and ^{3}H-thymidine, respectively.

EXAMPLE 24

Molecular Identity of GCC

Western blots from glioma membranes were obtained and probed with chlorotoxin-biotin. With this approach, a ~70 kD protein band (FIG. **20**) was identified. This band was also recognized by an antibody specifically generated to CLC-5, a chloride channel expressed in the kidney (Sakamoto et al. 1996; Steinmeyer et al. 1995). (This antibody was kindly provided by Drs. Jentsch and Guenther). Immunopercipitation with CLC-5 antibodies and subsequent probing with Ctx-biotin identified the same 70 kD band suggesting that GCC must have a high homology to CLC-5.

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

What is claimed is:

1. A pharmaceutical composition comprising chlorotoxin wherein the pharmaceutical composition is suitable for use in humans.

2. The composition of claim **1** wherein the chlorotoxin is selected from the group consisting of recombinant chlorotoxin, synthetic chlorotoxin and native chlorotoxin.

3. The composition of claim **1** wherein the chlorotoxin is recombinant chlorotoxin.

4. The composition of claim **1** wherein the chlorotoxin is labeled.

5. The composition of claim **4** wherein the chlorotoxin label is a radiolabel.

6. The composition of claim **5** wherein the chlorotoxin radiolabel is selected from the group consisting of ^{131}I and ^{125}I.

7. The composition of claim **4** wherein the chlorotoxin label is a fluorescent moiety.

8. A pharmaceutical composition comprising a pharmacologically effective dose of chlorotoxin and a cytotoxic moiety that is effective to treat an individual having a glioma or meningioma.

9. A pharmaceutical composition comprising a pharmacologically effective dose of chlorotoxin and a cytotoxic moiety that is effective to suppress the growth of tumor cells which are glial in origin.

* * * * *

US005223253A

United States Patent [19]

Hall et al.

[11] Patent Number: 5,223,253

[45] Date of Patent: Jun. 29, 1993

[54] BOVINE VACCINE COMPOSITIONS AND METHOD FOR PREVENTING TRICHOMONAS INFECTIONS USING SAME

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[21] Appl. No.: 411,921

[22] Filed: Sep. 28, 1989

[51] Int. Cl.[5] A61K 39/00; A01N 63/00

[52] U.S. Cl. 424/88; 424/92; 424/450; 530/350; 435/252.1

[58] Field of Search 424/88, 93, 92, 450; 530/350; 435/252.1

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[57] ABSTRACT

This invention provides a bovine vaccine composition comprising an immunogenically active component having inactivated bovine Trichomonas cells or antigens derived therefrom, in combination with an effective amount of an immunogenically suitable adjuvant; and a veterinary pharmaceutically acceptable carrier or diluent. The vaccine composition is useful to prevent Tritrichomonas (Trichomonas), e.g., *T. foetus*, infection in bovine, and may also be combined with other vaccine compositions or therapy. A method for preventing Trichomonas infection in bovine is also provided.

6 Claims, No Drawings

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BOVINE VACCINE COMPOSITIONS AND METHOD FOR PREVENTING TRICHOMONAS INFECTIONS USING SAME

The present invention provides a bovine vaccine composition and a method for preventing Trichomonas infections in bovine using such vaccine composition. More particularly, this invention relates to a bovine vaccine composition comprising an immunologically active component, i.e., inactivated bovine Trichomonas cells or antigens derived therefrom in combination with an effective amount of an immunogenically stimulating adjuvant, and veterinary pharmaceutically acceptable carriers or diluents therefor.

BACKGROUND OF THE INVENTION

Trichomoniasis is a venerally transmitted disease of cattle which may cause infertility, early and late abortions or uterine infections, further resulting in varying degrees of reproductive inefficiency. The causative agent for trichomoniasis is the parasitical protozoan, Tritrichomonas (Trichomonas) e.g., *T. foetus.*

Trichomoniasis is not a new disease but reported incidence has increased due to improved diagnostic procedures. Movement of cattle from infected areas has also expanded the incidence of the disease. Since there are no viable signs of trichomoniasis, it may go undetected until a producer becomes alarmed by such signs as cows returning to heat at the end of the breeding period, calves of varying ages and sizes at weaning, or a strung-out calving season, and, in response to such signs, the producer takes a critical look at his herd's reproductive efficiency.

The disease is highly transmissible. One infected bull results in herd infection, since a bull infects 80–90% of the cows he services. This can result in calf crop reductions as high as 40% or even more. Losses due to *T. foetus* infection are estimated to be in excess of $500 million annually. One hundred cow herd losses can total $16,000–$20,000.

The *T. foetus* is a protozoan that lives in the crypts (wrinkles or folds) on the mucosal surface of the penis and prepuce of the bull. These organisms are transmitted to the cow only by breeding. If the cow is exposed to *T. foetus* at the time of breeding, these tiny protozoa grow and multiply on the lining of the uterus, causing an inflammation (metritis) which eventually disrupts the placental circulation supplying nutrients to the embryo and results in death of the embryo within the first 30–60 days of pregnancy. Since the embryo is so small, the only outward sign may be a uterine infection with minimal vaginal discharge. Carrier cows do exist, which further complicates *T. foetus* bovine infection in cows. Typically, these chronically infected cows carry a calf to term while infected with the *T. foetus* organism. Clean bulls bred to carrier cows can result in the infection of the entire herd. Culling bulls will do no good so long as carrier cows remain in the herd.

The infection in the bull is completely without symptoms. Although young bulls may become infected, they are relatively resistant due to the lack of crypts on the mucous lining of the penis and prepuce. These folds in the mucous lining provide the necessary environment for the replication of the *T. foetus*. As a bull matures (usually at 2½ to 3 years), these crypts become more pronounced, providing a more suitable environment for the *T. foetus*. While the Trichomonads grow in these crypts, they do not stimulate the bull's immune system, thus the bull remains infected.

Past attempts to immunize or vaccinate cattle against Trichomonas infection have not been very successful for a variety of reasons, including any or all of the following: 1) vaccines developed for infected bulls failed to clear or prevent infection in older bulls; 2) vaccines developed for cows failed to prevent infection in bulls; 3) vaccines developed for cows failed to stimulate local immunity in the cervico-vaginal cavity.

It is an object of the present invention to provide an effective bovine vaccine composition having high antigen load and including a very potent adjuvant which will stimulate localized mucosal immunity against *Trichomonas foetus* when administered systemicly to a subject bovine.

It is another object of the present invention to reduce the incidence of abortion among cows susceptible to Trichomonas infection.

It is still yet another object of this invention to increase reproductive or breeding efficiency among bovine animals (bulls and cows) susceptible to Trichomonas infection.

Yet another object of the present invention is to provide a method for preventing Trichomonas infection in cattle by immunizing these animals with an efficacious vaccine composition.

These and other objects will become more apparent in light of the detailed description which follows.

SUMMARY OF THE INVENTION

The present inventors have unexpectedly discovered that an immunogenically active component can be made and usefully incorporated into a vaccine composition for preventing Trichomonas infections in bovine, i.e., cattle, cows, bulls, heifers. The immunogenically active component has inactivated, e.g., chemically inactivated, bovine Tritrichomonas (Trichomonas) cells or antigens derived therefrom, such as outer membrane extracted antigen. The immunogenically active component is combined with an effective amount of an immunogenically stimulating adjuvant, and a veterinary pharmaceutically acceptable carrier or diluent therefor.

DETAILED DESCRIPTION OF THE INVENTION

All literature references, patents and patent applications cited in this specification are hereby incorporated by reference in their entirety.

The present invention provides a vaccine composition comprising an immunogenically active component having inactivated bovine Trichomonas cells, e.g. *T. foetus* or antigens derived therefrom in combination with an effective amount of an immunogenically stimulating adjuvant; and a veterinary pharmaceutically acceptable carrier or diluent therefor.

As used herein, the term "immunogenically active" component refers to the ability of the component described herein to stimulate an immune response, i.e., to cause the production of antibodies and/or cell-mediated response when introduced into a subject (mammal, e.g., bovine). More specifically, the term "immunogenically active" component refers to the ability of this component to stimulate secretory antibody and/or cell-mediated response production in local mucosal regions, e.g., cervico-vaginal cavity, when administered systemically as a vaccine composition according to the present invention.

The Trichomonas cells, e.g., *T. foetus* cells, or antigens derived therefrom which are used to make the immunogenically active component of the vaccine composition can be isolated from the fluids or tissues of mammalian, e.g., bovine (bulls, cows), sources or specimens obtained from animals infected with the Trichomonas protozoan. Such sources or specimens include, for example, vaginal, cervical, uterine, prepuce, scrapings and secretions. In particular, the Trichomonas protozoans can be isolated from the vaginal, cervical and uterine fluids from infected cows which have aborted calves, and also from the preputial sheath from infected bulls. The Trichomonas protozoans can be maintained in the infected bovine, or in suitable nutrient media known in the art, such as, for example, Diamond's modified medium which is prepared by the method described by Diamond, L. S., (1983) Human dwelling protozoa: Entamoeba, Trichomonads and Grandes. In *In Vitro Cultivation of Protozoan Parasites*, edited by J. B. Jensen, CRC, Boca Raton, pp. 65-109 (not more than 10% serum protein is added to the Diamond medium).

Diamond's medium contains the following composition:

	Gm/Liter
Casein Hydrolysate	20.0-50.0*
Yeast Extract	10.0-50.0*
Maltose	5.0
L-Cysteine Hydrochloride	1.0
L-Ascorbic Acid	0.2
Dibasic Potassium Phosphate (anhydrous)	0.8
Monobasic Potassium Phosphate (anhydrous)	0.8

*Batch to batch variation influences growth of the organism. Concentration levels vary according to the batch of new material and growth obtained with the organism.

The powdered media is dissolved in deionized water and the media is then sterilized by autoclaving at 121° C. The media is labeled and may be stored at room temperature until use. To the prepared Diamond's medium, as noted above, not more than 10% serum protein is added.

The Trichomonas protozoans can be isolated from the fluids or tissues as described above, e.g., vaginal fluid, or preputial washings of infected bovine animals and cultured in suitable nutrient media. The Trichomonas protozoans can be separated from the culture media using techniques well-known in the art, such as centrifugation, filtration and the like.

Following their separation as whole cell isolates, the Trichomonas protozoans can be inactivated by conventional inactivation means known in the art. For example, chemical inactivation of the Trichomonas whole cell isolates can be carried out by contacting the cells with a chemical inactivating agent. Such agents include by way of non-limiting example, binary ethylenimine, beta-propriolactone, formalin, merthiolate, glutaraldehyde, sodium dodecyl sulfate, Triton-100, or a combination of any of these agents in an aqueous suspension. Preferred as a chemical inactivating agent is merthiolate at a final concentration of 1:10,000 for 24 to 72 hours.

The Trichomonas cellular isolates can also be inactivated by heat or psoralen in the presence of ultraviolet (UV) light.

After inactivation, the inactivated Trichomonas whole cells can be adjusted to an appropriate concentration of from about 10^8 to about 10^9 in combination with an immunogenically stimulating adjuvant. When antigens derived from Tritrichomons (Trichomonas) cells, e.g., *T. foetus* cells, are employed, a suitable amount of protein or antigen per dose may be used, for example, 50 to 100 ug/dose.

As used herein the term "immunogenically stimulating adjuvant " refers to an agent, compound or the like, which potentiates or stimulates the immune response in a subject animal when administered in combination with the inactivated whole cells. Thus, the immune response, elicited by the inactivated whole cell-adjuvant combination, as measured by antibody and/or cell-mediated response, will generally be greater than that provoked by the inactivated whole cells alone.

The immunogenically stimulating adjuvants augment the immune response provoked by the inactivated Trichomonas cells. The inactivated Trichomonas cells may or may not elicit a desired immune response, e.g., a local mucosal, e.g., vaginal, immunity, when systemically administered alone. An essential feature of the present invention is the combination of the inactivated Trichomonas cells and immunogenically stimulating adjuvant, which provide the desired immune response.

Non-limiting examples of the immunogenically stimulating adjuvants used in the practice of the present invention are surfactants, e.g., hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dioctadecyl-N'-N-bis(2-hydroxyethylpropane diamine), methoxyhexa-decylglycerol and pluronic polyols, saponin, Quil A; polyanions, e.g., pyran, dextran sulfate, poly IC (polynucleotide complex of polyinosinic-polycytidylic acid) polyacrylic acid, carbopol, aluminum hydroxide, aluminum phosphate; peptides, e.g., muramyl dipeptide, dimethylglycine, tuftsin; oil emulsions, immunomodulators, e.g., interleukin-1, interleukin-2; or combinations of any of the foregoing adjuvant agents. Preferred as an immunogenically stimulating adjuvant is an adjuvant containing, for example, 1-20% by volume of an oil-in-water emulsion and 0.1-10 ug chlorotoxin or 0.5 to 10 mg saponin per dose, whether such dose is 2- or 5- or 10 ml/dose.

It has been discovered that the adjuvants described above will act in effective amounts to immunogenically stimulate the inactivated Trichomonas cells or antigens derived therefrom when combined therewith, to form the active component of the vaccine composition of this invention. As used herein, the effective amount of the immunogenically stimulating adjuvant can comprise from about 1% to about 50%, preferably from about 5% to about 20%.

The vaccine composition of the present invention further comprises a veterinary pharmaceutically acceptable carrier or diluent. Such a carrier or diluent useful in the practice of the present invention is saline. Preferred is saline, e.g., 2.0 mL saline.

As further embodiments of the present invention, the vaccine composition can be administered, for example, by incorporating the active component into liposomes. Liposome technology is well-known in the art having been described by Allison, A. C. and Gregoriades, G., Liposomes as immunologic adjuvants. *Nature* 252:252-54 (1974); and Dancy, G. F., Yasuda, T., and Kinsky, S. C., Effect of liposomal model membrane composition on immunogenicity. *J. Immunol.* 120:1109-13 (1978). In addition, the active component can be conjugated to suitable biological compounds or materials, such as, for example, polysaccharides, peptides, proteins, or a combination of any of the foregoing. Conjugated vaccines are described by Coon, J., and

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It is advantageous to formulate the vaccine composition of this invention in dosage unit form to facilitate administration and insure uniformity of dosage. Thus, in another embodiment, this vaccine composition can be formulated in dosage unit form comprising at least about 1×10^6 inactivated Trichomonas cells, preferably at least about 5.0×10^7 cells.

In a further embodiment, the vaccine composition can comprise a parenteral injectable form, again to ease its administration to a subject bovine.

The present invention provides a method for preventing Trichomonas infection in bovine comprising administering to a bovine in need of such prevention an effective amount of the vaccine composition described above.

The routes of administration contemplated by the present invention are parenteral, e.g., subcutaneous, intramuscular, intraperitoneal and intradermal. Preferred routes of administration are subcutaneous and intramuscular.

It has been discovered that the vaccine composition of the present invention is useful to prevent Trichomonas infection in bovine that need such protection when administered parenterally, e.g., subcutaneously or intramuscularly, in effective amounts and according to a schedule dictated by the breeding of the bovine. For example, the vaccine composition has been found to be effective in preventing Trichomonas infection when the final dose is administered at least about fourteen (14) days before breeding of the treated animal. In this way, the treated animal has time to build immunity prior to breeding. An effective regimen of treatment includes administering the vaccine composition, for example, in dosage unit form as described above, at least about two times, with each administration separated by about two (2) to about four (4) weeks, i.e., from about fourteen (14) to about thirty (30) days or so.

A preferred treatment schedule would include parenteral administration, e.g., subcutaneous or intramuscular injection, at least about 4–6 weeks prior to breeding. Because at least two administrations (injections) are preferred, these administrations (injections) could be given, for example, at about six (6) weeks and about two (2) weeks, respectively, before breeding of the treated animal.

This invention provides a multi-vaccine composition comprising the vaccine composition as described above, and at least one vaccine composition directed against a pathogen selected from the group consisting of *Leptospira canicola, L. icterohaemorrhagiae, L. pomona, L. hardjo, L. grippotyphosa* and *Campylobacter fetus*, or a combination or any of the foregoing.

The working examples set forth below are intended to illustrate the invention without limiting its scope.

EXAMPLE 1

Vaccine Preparation

A bovine vaginal isolate of *T. foetus* was obtained from the vaginal fluids of a cow which had been naturally infected with *Trichomonas foetus* and which had recently aborted a calf. The isolate was cultivated in a modified Diamond's medium at 37° C. in air to a density of 1×10^7 protozoans/mL. The modified medium was prepared from the following composition:

	Gm/Liter
Casein Hydrolysate	20.0–50.0
Yeast Extract	10.0–50.0
Maltose	5.0
L-Cysteine Hydrochloride	1.0
L-Ascorbic Acid	0.2
Dibasic Potassium Phosphate (anhydrous)	0.8
Monobasic Potassium Phosphate (anhydrous)	0.8

The powdered media was dissolved in deionized water and the media was then sterilized by autoclaving at 121° C. To the prepared Diamond's medium not more than 10% serum protein was added.

Trichomonas foetus protozoans were harvested by centrifugation, resuspended in a small volume of saline adjusted to pH 7.2. Merthiolate was added to this solution to a final concentration of 0.0001% and incubated at 4° C. for 24 hours.

The vaccine was formulated by suspending the appropriate volume of inactivated cells in an adjuvant containing 1–20% by volume of an oil-in-water emulsion and 0.5 to 10 mg saponin per 2 mL dose.

EXAMPLE 2

Challenge and Isolation of *T. foetus*

Fourteen to 35 days after vaccination i.e., following second vaccination, challenge was initiated. 120 heifers were challenged by breeding with persistently infected bulls and/or intra-vaginal infusion by 1×10^8 protozoans by an infectious isolate of *T. foetus* which had not been subcultured more than four times. Mucous from the reproductive tract of each heifer was sampled thirty (30) days post breeding and weekly thereafter *T. foetus* was isolated at 37° C. in screw capped tubes (15×20 mm) containing 10.0 mL of modified Diamond's medium (prepared to according to Example 1) without agar. Cultures were held 12–14 days before isolation attempts were determined negative.

EXAMPLE 3

Antibody Response to Subcutaneous Injection of Vaccine

60 heifers were injected subcutaneously with a vaccine prepared according to Example 1. The heifers received two injections of vaccine (5×10^7 whole cells in 2.0 mL saline containing 1–20% oil-in-water emulsion and 0.5–10 mg of saponin at 14 day and 21 day intervals. The antibody levels were determined by indirect immunofluorescent antibody (IFA) assay conducted as follows: Slides were prepared using *T. foetus* organisms washed two to three times in PBS and resuspended in PBS. Slides were air dried before fixing in chilled acetone. Slides were then flooded for 30 minutes at 37° C. with two-fold dilutions of test sera before rinsing in PBS. A dilution of FITC conjugated anti IgG sera was then added to each slide. The slides were incubated at 37° C. for 30 minutes, washed in PBS, mounted and read microscopically using a fluorescent microscope. The antibody levels were also determined by a culture protection i.e., serum neutralization (SN) assay conducted as follows: Two-fold dilutions of test sera were incubated with a quantitated amount of viable *T. foetus*. Neutralization was determined by cultivating the serum-organism mixtures in confluent MDBK monolayers. Viable *T. foetus* will cause a characteristic cytopathic effect to the monolayer and neutralized *T. foetus*

will cause no cytopathic effect. The results of these assays are summarized in Table 1 below.

TABLE 1*

Time (Days Following Vaccinations (V_1-V_2)	Protection					
	Vaccinates		Controls		Freunds	
	IFA	SN	IFA	SN	IFA	SN
V_1 (0 days)	5	34	_5	32	_5	32
V_2 (28 days)	746	104	_5	32	264	74
28 day post V_2	1715	588	_5	32	528	446
75 days post challenge	264	52	_5	32	606	74

*Trichomonas appears sensitive to non-immune bactericidal factors in serum diluted 1:32 or less.

The data summarized in Table 1 indicates that all the heifers, that were vaccinated according to the present invention, developed humoral IFA and SN antibody titers following the first vaccination and an anamnestic response following the second vaccination.

The significance in protection of humoral antibody stimulation has been questioned by some investigators. It has been demonstrated by at least one study that vaginal/uterine infection by a pathogen does not stimulate serum antibody. (S. Skirrow, R. Bon Durant, Abstract #214, Conference of Research Workers in Animal Disease, 1988, p. 38) Systemic immunization can stimulate significant local antibody in the vagina. Significant correlation has been demonstrated with the cell-mediated immune (CMI) response. In any event, as shown by the results in Table 1, the immune response elicited by the immunogenicity serial performed in accordance with this invention was more effective than Freund's antibody titer following primary vaccination, as well as an anamnestic response. Therefore, the duration and intensity of local immunity conferred has been found to increase by using multiple vaccinations. A vaccine comprising inactivated *T. foetus* has also been found effective to actively immunize susceptible cattle against trichomoniasis.

Trichomonas was recovered from 9 to 10 non-vaccinated controls; recovery rate from two heifers was 12 weeks, from one heifer each for 11 weeks, 10 weeks, 8 weeks, 5 weeks and 3 weeks, respectively. Trichomonas was also recovered from two heifers for one week. A total of 84 samples were taken from the control group through the end of Month 3 and 104 samples through the end of detectable infection as designated by five consecutive negative cultures.

The results of the recovery procedure, as evidence of the efficacy of the vaccine composition of the present invention, are illustrated in Table 2 below.

TABLE 2

Trichomonas Recovery from Vaccinated and Non-Vaccinated Calves Following Exposure to Infected Bulls and Artificial Infusion

Calf No.	Month 1		Month 2				Month					Month 4			
	22	28*	06*	13	20*	27	08	10	17	24	31	07	14	25	18
Vaccinates															
9	–	–	–	–	–	–	–	–	–	–	–				
13	–	–	–	–	–	–	–	–	–	–	–				
17			–	+	+	–	–	–	–	–	–				
37				–	–	–	–	–	–	–	–				
42				–	–	–	–	–	–	–	–				
57				–	+	+	–	–	–	–	–				
61				–	–	–	–	–	–	–	–				
63				–	–	–	–	–	–	–	–				
92				–	–	+	–	–	–	–	–				
111				–	–	–	–	–	–	–	–				
Total samples through end of Month 3 = 87% = 5.7%, i.e., 5.7% of all samples from vaccinated heifers were positive for Trichomonas infection.															
Control															
18			–	–	+	–	–	–	–						
29	–	–	–	–	–	+	–	–	–	–	–				
40				+	–	+	+	–	–	–	–	–	–	–	–
51				–	–	–	–	–	–	–	–	–		died Month 4, Day 26	
52				+	+	+	+	+	+	+	+	+	+	+	+
56				+	+	–	+	+	+	+	+	+	+	+	+
59				+	+	+	+	+	+	+	+	+	+	+	+
83				–	–	–	+	–	+	–	–	+	+	+	
89				+	–	+	+	+	+	+	+	+	+	+	
95				+	–	–	+	+	+	+	+	+	+	–	–

Artificial Infusion:
Month 1, Day 29;
Month 2, Day 6; and
Month 2, Day 20.
Total samples through end of Month 3 = 84% = 51.2%, i.e., 51.2% of all samples from non-vaccinated controls were positive.
Total samples through Month 4, Day 25 = 104% = 60.6%, i.e., 60.6% of all samples taken from controls through month 4 were positive.

Statistical Analysis: t-Test; = 18, t = 3.72, p = 0.001.

As shown by the results in Table 2, the percent positive cultures were 51.1 and 60.6, respectively. The average number of days of positive isolation was 44.1 days. These results indicate that 1) 70% of the controls exhibited an infection characterized by positive isolation on three sampling days as compared to none, i.e., 0%, of the vaccinates; 2) there was a 95.2% reduction in the number of positive samples. Therefore, following infusion, the heifers were successfully exposed to Trichomonas and colonization did occur in the controls. Recovery of Trichomonas from the vaccinates was significantly reduced from the challenge control heifers (p=0.001).

The results in Table 2 also indicate that it is more difficult to initially establish an infection in the vaccinates compared to the controls after each cycle in infusion in the heifers; two consecutive weeks from two heifers and one week from one heifer. It is significant that 100% of the vaccinates failed to demonstrate a chronic infective state. A total of 87 samples were taken from the vaccinated group through the end of Month 3 and only 5.7% of these samples were positive. The average number of days of positive isolation was 2.1.

From the results obtained in Example 3 above, particularly Table 2, it can be concluded that vaccination according to the present invention, decreases the ability of the infectious challenge Trichomonas organism to colonize the bovine reproductive tract. It may also be concluded that vaccination according to the present invention increases the clearance of the infectious Trichomonas organism from the bovine reproductive tract.

EXAMPLE 4

Protection Against *T. foetus* Induced Abortion

Thirty-four 18 month old holstein heifers were assigned to control (12), soluble vaccine (11) and whole vaccine (11) groups to determine the effect of Trichomonas foetus vaccines on the reproductive performance of *T. foetus* infected animals. Heifers were bred to *T. foetus* infected bulls beginning two weeks after the second *T. foetus* vaccination. All immunized animals developed antibody titers of at least 1:1,000 following vaccination. In addition, all control and immunized animals became infected with *T. foetus*. However, the duration of infection was approximately two weeks shorter in immunized animals. Approximately 42% (5 of 12) of the control heifers remained *T. foetus* infected for the duration of the experiment, while only 18% (2 of 11) of each of the vaccine groups remained infected for the duration of the experiment. Finally, 27% (3 of 11) of the heifers in each of the vaccine groups were pregnant at slaughter, while none of the control heifers were pregnant at slaughter.

The efficacy of the *T. foetus*-based vaccines of the present invention can be increased by employing immunogenic fractions derived therefrom by methods which are well known in the art. For example, soluble Trichomonas outer envelope antigens which surround the protoplasmic cylinder of protozoans can be readily extracted as disclosed by Kvasnicka, W. G., Taylor, R.E.L., Hands, D., Huang, J.-C. and M. R. Hall, "An Assessment of the Efficacy of Immunization of Cattle with Vaccines Containing *Trichomonas foetus*, 9th Annual Food-Animal Disease Research Conference (1988), Pullman, W. A., which serve to increase immunogenicity.

This fraction may provide immunogens which impart an equal or greater resistance to Trichomonas infection when employed as the active component of a bovine vaccine composition in accordance with the present invention.

This invention has been described with reference to various specific and preferred embodiments and techniques. It should be understood, however, that many variations and modifications can be made by those of ordinary skill in the art while remaining within the spirit and scope of the invention.

What is claimed is:

1. A method of preventing Trichomonas infection in a cow or heifer comprising administering to said cow or heifer an effective amount of the vaccine composition comprising an immunogenically active component having
   - from 1×10^6 to 1×10^9 of inactivated bovine Trichomonas cells or antigens derived therefrom per dose in combination with an effective amount of an immunogenically stimulating adjuvant; and
   - a veterinary pharmaceutically acceptable carrier or diluent therefor.

2. The method according to claim 1, wherein said vaccine composition is parenterally administered.

3. The method according to claim 2, wherein said parenteral administration is carried out subcutaneously.

4. The method according to claim 2, wherein said parenteral administration is carried out intramuscularly.

5. The method according to claim 1, wherein said vaccine composition is administered at least fourteen days before breeding of said cow or heifer.

6. The method according to claim 5, wherein said vaccine composition is administered at least two times, each administration separated by 14 to 30 days.

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